PATENT
Docket No.: **19603/4230 (CRF D-2238-04)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	8339	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR § 41.37, appellants hereby file their appeal brief. Enclosed is the filing fee of $250.00 required by 37 CFR § 41.20(b)(2). You are hereby authorized to charge/credit Account No. 14-1138 for any deficiency/overage.

I. REAL PARTY IN INTEREST

Cornell Research Foundation, Inc., as assignee of U.S. Patent Application Serial No. 09/282,239, is the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences pertaining to the above-identified application.

III. STATUS OF CLAIMS

A. Claims 25, 26, and 29 Are Finally Rejected

Claims 25, 26, and 29 have been finally rejected under 35 U.S.C. § 102(e) as anticipated by or, in the alternative, under 35 U.S.C. § 103(a) for obviousness over U.S. Patent No. 6,361,996 B1 to Rao et al. ("the '996 patent").

B. Claims 1-24 and 27-28 Have Been Canceled

Claims 1-24 and 27-28 have been canceled.

C. No Claims Stand Allowed

No claims stand allowed.

D. Claims 25, 26, and 29 Are On Appeal

The decision of the examiner finally rejecting claims 25, 26, and 29 is appealed. These claims, in their currently pending form, are set forth in the attached Claims Appendix.

IV. STATUS OF AMENDMENTS

There are no amendments pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed to an enriched or purified preparation of human mitotic oligodendrocyte-specified progenitor cells, where the mitotic oligodendrocyte-specified progenitor cells are from a post-natal human and a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of

the enriched or purified preparation (U.S. Patent Application Serial No. 09/282,239 ("the '239 Application") p. 14, line 24 to p. 15, line 2; p. 22, lines 6-17; and originally-filed claim 17). The present invention is also directed to an enriched or purified preparation of human mitotic oligodendrocyte progenitor cells, where the mitotic oligodendrocyte progenitor cells are from an adult human and a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of the enriched or purified preparation (the '239 Application p. 14, line 24 to p. 15, line 2 and p. 22, lines 6-17). The present invention is also directed to an enriched or purified preparation of human mitotic oligodendrocyte-specified progenitor cells, where a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of the enriched or purified preparation (the '239 Application p. 14, line 24 to p. 15, line 2 and p. 22, lines 6-17).

VI. GROUNDS OF REJECTION TO BE REVIEWED UPON APPEAL

Whether claims 25, 26, and 29 are properly rejected under 35 U.S.C. § 102(e) as anticipated by or, in the alternative, under 35 U.S.C. § 103(a) for obviousness over the '996 patent, when the '996 patent fails to teach or suggest each and every aspect of the claimed invention.

VII. ARGUMENT

A. Applicable Law

1. 35 U.S.C. § 102(e)

35 U.S.C. § 102(e) imposes the requirement that a claimed invention, to be patentable, must not have been "described in . . . a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent." To be anticipatory, under 35 U.S.C. § 102, a single prior art reference must disclose, either expressly or inherently, each limitation of the claim. *Minn. Mining & Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc.*, 976 F.2d 1559, 1565, 24 USPQ2d 1321, 1326 (Fed. Cir. 1992).

2. 35 U.S.C. § 103(a)

35 U.S.C. § 103 imposes the requirement that an invention, to be patentable, must not have been obvious over the prior art "at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." A proper *prima facie* showing of obviousness requires the U.S. Patent and Trademark Office ("PTO") to satisfy three requirements. First, the prior art itself must suggest the desirability and, therefore, obviousness of a modification of a reference or the combination of references to achieve a claimed invention. *See Hodosh v. Block Drug Co.*, 786 F.2d 1136, 1143 n.5, 229 USPQ 182, 187 n.5 (Fed. Cir. 1986); *In re Gordon*, 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984); *see also In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). Second, the PTO must show that, at the time the invention was made, the proposed modification had a reasonable expectation of success. *See Amgen v. Chugai Pharm. Co.*, 927 F.2d 1200, 1209, 18 USPQ2d 1016, 1023 (Fed. Cir. 1991). Finally, the combination of references must teach or suggest each and every limitation of the claimed invention. *See In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970).

Further, the question of obviousness should be analyzed in light of the holding of *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966) which sets forth the following factors for determining obviousness: (1) the scope and content of the prior art; (2) differences between the prior art and the claims at issue; (3) the level of ordinary skill in the pertinent art; and (4) such objective evidence of non-obviousness as commercial success, long felt but unresolved needs, and failure of others. All evidence must be weighed before reaching a conclusion on obviousness under § 103. *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561, 1564, 1 USPQ2d 1593, 1594 (Fed. Cir. 1987); *Hodosh v. Block Drug*, 786 F.2d at 1143, 229 USPQ at 187-88.

B. The Rejection of Claims 25, 26, and 29 Under 35 U.S.C. § 102(e) As Anticipated By Or, In the Alternative, Under 35 U.S.C. § 103(a) for Obviousness Over the '996 Patent Is Improper.

1. Background

The damaged brain is largely incapable of functionally significant structural self-repair (the '239 Application pp. 1-3 "Background of the Invention"). This is due in part to the apparent failure of the mature brain to generate new neurons (*Id.*). However, the absence of neuronal production in the adult vertebrate forebrain appears to reflect not a lack of appropriate neuronal precursors, but rather their tonic inhibition and/or lack of post-mitotic trophic and migratory support (*Id.*). Converging lines of evidence now support the contention that neuronal and glial precursor cells are distributed widely throughout the ventricular subependymal of the adult vertebrate forebrain, persisting across a wide range of species groups (*Id.*). Most studies have found that the principal source of these precursors is the ventricular zone, though competent neural precursors have been obtained from parenchymal sites as well (*Id.*). In general, adult progenitors respond to epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) with proliferative expansion, may be multipotential, and persist throughout life (*Id.*). In rodents and humans, their neuronal daughter cells can be supported by brain-derived neurotrophic factor (BDNF), and become fully functional *in vitro*, like their avian counterparts (*Id.*).

A major impediment to both the analysis of the biology of adult neural precursors, and to their use in engraftment and transplantation studies, has been their relative scarcity in adult brain tissue, and their consequent low yield when harvested by enzymatic dissociation and purification techniques (*Id.*). As a result, attempts at either manipulating single adult-derived precursors or enriching them for therapeutic replacement have been difficult (*Id.*). The few reported successes at harvesting these cells from dissociates of adult brain, whether using avian, murine, or human tissue, have all reported <1% cell survival (*Id.*). Thus, several groups have taken the approach of raising lines derived from single isolated precursors, continuously exposed to mitogens in serum-free suspension culture (*Id.*). As a result, however, many of the basic studies of differentiation and growth control in the neural precursor population have been based

upon small numbers of founder cells, passaged greatly over prolonged periods of time, under constant mitogenic stimulation (*Id.*). The phenotypic potential, transformation state and karyotype of these cells are all uncertain; after repetitive passage, it is unclear whether such precursor lines remain biologically representative of their parental precursors, or instead become transformants with perturbed growth and lineage control (*Id.*).

In order to devise a more efficient means of isolating native, unpassaged and untransformed progenitor cells from brain tissue, a strategy by which brain cells could be freely dissociated from brain tissue, then transduced *in vitro* with plasmid DNA bearing a fluorescent reporter gene under the control of neural progenitor cell-type specific promoters was developed (*Id.*). This permitted isolation of the elusive neuronal progenitor cell of the CNS, using the Tα1 tubulin promoter, a regulatory sequence expressed only in neuronal progenitor cells and young neurons (*Id.*).

The repair of damaged brain requires not only sources of new neurons but also new glial support cells (*Id.*). Oligodendrocytes are the glial cell type that produce myelin and insulate neuronal axons by ensheathment with myelin-bearing cell processes (*Id.*). Like neurons, oligodendrocytes are largely postmitotic and cannot regenerate through proliferative expansion (*Id.*). However, persistent oligodendrocyte progenitors have been described in adult rodent subcortical white matter, and may provide a substrate for remyelination after demyelinating injury (*Id.*). In humans, the demonstration and identification of analogous subcortical oligodendrocyte progenitor cells has been problematic (*Id.*). A pre-oligodendrocytic phenotype has been described in adult human subcortical white matter, though these postmitotic cells may have included mature oligodendrocytes recapitulating their developmental program after dissociation (*Id.*). Rare examples of oligodendrocytes derived from mitotic division have also been reported in human subcortical dissociates, but the identification and isolation of their mitotic progenitors have proven elusive (*Id.*). As a result, the enrichment of these cells for functional utilization has proven difficult (*Id.*). In particular, the cells have not been preparable in the numbers or purity required for *in vivo* engraftment into demyelinated recipient brain, whether experimentally or for clinical therapeutic purposes (*Id.*).

A strong need therefore exists for a new strategy for identifying, separating, isolating and purifying native oligodendrocyte precursor cells from brain tissue (*Id.*). Such isolated, enriched native precursors may be used in engraftment and transplantation in demyelinating disorders, as well as for studies of growth control and functional integration (*Id.*).

2. Description of the '996 Patent

The '996 patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells (the '996 patent Abstract). The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons (*Id.*). The '996 patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added) (the '996 patent col. 23, lines 1-5).

In the outstanding office action, the sole portion of the '996 patent specifically identified in support of the rejection is Figure 1 (see p. 3, lines 3-8) and the supporting text regarding cell type 14. As this figure and the paragraph bridging columns 6 and 7 make clear, cell type 14 is a multipotential precursor cell that can generate oligodendrocytes 18 and astrocytes 22. Cell type 14 is said to be generated from embryonic spinal cord stem cells. While noting that the '996 patent's experimental work was with rat cells, the outstanding office action states that human stem cells could likewise be isolated and, presumably used to produce human multipotential precursors of both oligodendrocytes and astrocytes. Even if this is true, one is still left to speculate how the '996 patent teaches the oligodendrocyte-specified progenitor cells of the claimed invention. The only possible bases for taking such a position are explicit anticipation, inherent anticipation, and obviousness. For the reasons noted *infra*, the '996 patent is not sufficient on any of these grounds.

3. The '996 Patent Does Not Explicitly Anticipate the Claimed Invention.

Nowhere does the '996 patent disclose the claimed enriched or purified preparations of human mitotic oligodendrocyte-specified and oligodendrocyte progenitor cells. Indeed, the astrocyte/oligodendrocyte precursor cells disclosed in the '996 patent are different from the oligodendrocyte-specified and oligodendrocyte progenitor cells claimed in the present application (Declaration of Mahendra S. Rao, M.D., Ph.D. Under 37 C.F.R. § 1.132 ("First Rao Declaration") ¶ 6 (attached hereto as Exhibit 1) and Second Declaration of Mahendra S. Rao, M.D., Ph.D. Under 37 C.F.R. § 1.132 ("Second Rao Declaration") ¶ 6 (attached hereto as Exhibit 2)).

Declarant Mahendra S. Rao ("Dr. Rao"), the same Dr. Rao who is co-inventor of the '996 patent, has stated that the '996 patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells claimed in the present patent application (*Id.*). Furthermore, one of Dr. Rao's publications (Rao et al., "Glial-Restricted Precursors are Derived from Multipotent Neuroepithelial Stem Cells," *Devel. Biol.* 188:48-63 (1997) (attached hereto as Exhibit 3)) clearly demonstrates that such A2B5+/NCAM- cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage (*Id.*).

As shown in Figures 1-2 of the '996 patent, the astrocyte/oligodendrocyte precursor cells 14 and 54, respectively, differentiate directly into two cell types—i.e., of astrocytes and oligodendrocytes (Second Rao Declaration ¶ 7). It is known from clonal analysis that there is a homogenous population of astrocyte/oligodendrocyte precursor cells in which individual cells generate oligodendrocytes and two kinds of astrocytes by the process described in the '996 patent (*Id.*). It is important to note that multiple pathways to generate post-mitotic, mature oligodendrocytes, have been described (*Id.*). Anderson and colleagues have shown that an oligodendrocyte/motorneuron precursor exists that does not make astrocytes (Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002) (attached hereto as Exhibit 4)) (*Id.*). Other investigators have shown distinct sites of origin of oligodendrocytes and astrocytes presumably from separate precursors

(Vallstedt et al., "Multiple Dorsoventral Origins of Oligodendrocyte Generation In the Spinal Cord and Hindbrain," *Neuron* 45:55-67 (2005) (attached hereto as Exhibit 5) and Cai et al., "Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005) (attached hereto as Exhibit 6)) (*Id.*). Yet other investigators have shown that different kinds of oligodendrocyte progenitors exist (Pringle et al., "*Fgfr3* Expression by Astrocytes and Their Precursors: Evidence that Astrocytes and Oligodendrocytes Originate In Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003) (attached hereto as Exhibit 7)) (*Id.*).

Dr. Rao is not aware of any evidence that the astrocyte/oligodendrocyte precursor cells of the '996 patent generated mature oligodendrocytes by way of an intermediate oligodendrocyte-specific precursor (*Id.*). Indeed, Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development In the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences In GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002) (attached hereto as Exhibit 8) have suggested that the '996 patent describes a glial progenitor that gives rise to a more restricted astrocyte/oligodendrocyte precursor that still directly makes predominantly astrocytes and a small minority of oligodendrocytes (*Id.*). Thus, cells in the '996 patent's pathway to oligodendrocyte production are bi-potential astrocyte/oligodendrocyte progenitor cells that have strong astrocytic bias (*Id.*). These cell types are very different from the claimed oligodendrocyte-specified progenitor cells of the present application (*Id.*).

Example 15 of the '996 patent specifically conducts work to investigate whether mature astrocytes and oligodendrocytes are generated from committed unipotential cells present in the A2B5+ population of cells (i.e. cell types 14 and 54 in Figures 1 and 2, respectively) or whether single cells are bipotential and can generate both astrocytes or oligodendrocytes. As a result of this investigation, the '996 patent concludes that the A2B5+ cells were at least bipotential and were restricted to glial cell lineages (see col. 21, lines 41-45 and col. 22, lines 9-11 of the '996 patent). Having conducted this experiment and reached these conclusions, it is apparent that the '996

patent not only fails to disclose the claimed oligodendrocyte-specified progenitor cells but teaches away from their presence.

In response to appellants' arguments, the examiner repeatedly states that appellants are arguing differences between the '996 patent disclosure and the disclosure of the present application—not the claims of the present application. From the foregoing, this is clearly not the case. As repeatedly noted above, it is appellants' position that the '996 patent fails to teach oligodendrocyte-specified progenitor cells. This is precisely what appellants are claiming, so it is clear that their arguments are fully commensurate in scope with the claimed invention. What remains unclear, however, is exactly how the examiner can say that the disclosure of the '996 patent teaches the claimed invention.

In addition to failing to teach the claimed oligodendrocyte progenitor cells, the '996 patent also worked with cells from rats rather than from humans, as required by the claimed invention. In particular, there are fundamental differences between the biology of rat and human oligodendrocyte progenitor cells (Declaration of Steven A. Goldman Under 37 C.F.R. § 1.132 ("First Goldman Declaration") ¶ 7 (attached hereto as Exhibit 9)[1]. Furthermore, there are fundamental differences between the lineage restriction and potential of neonatal and adult oligodendrocyte progenitor cells (Noble et al., "The O2A (Adult) Progenitor Cell: A Glial Stem Cell of the Adult Central Nervous System," *Seminars in Cell Biol.* 3:413-22 (1992) (attached hereto as Exhibit 23) and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cells Effectively Myelinate Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004) (attached hereto as Exhibit 18) (Third Goldman Declaration ¶ 6). These biological differences between both rat and human and perinatal and adult progenitor cells were not recognized by the '996 patent, whose cells were restricted to neonatal rodent derivation (First Goldman Declaration ¶ 7; Third Goldman Declaration ¶ 6). Whereas rat oligodendrocytes appear to retain mitotic potential, human oligodendrocytes do not (Third Goldman Declaration ¶ 7). *See* Kirschenbaum et al., "*In Vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain," *Cerebral Cortex* 6:576-89

[1]Although the First Goldman Declaration was directed to U.S. Patent No. 5,276,145 to Bottenstein ("Bottenstein"), the issues regarding that reference are substantially the same as those pertaining to the '996 patent.

(1994) (attached hereto as Exhibit 11). As a result, the oligodendrocyte progenitor cells of the rat brain cannot be considered homologous to its human counterpart (*Id.*). In particular, methods that permit the selective extraction and/or growth of oligodendrocyte progenitors from the rat brain do not differentiate between oligodendrocyte progenitor cells and mature oligodendrocytes able to re-enter the mitotic cycle (*Id.*). In humans, these constitute two discrete phenotypes, lineally related but temporally distinct (*Id.*). The present invention teaches the selective acquisition of a highly enriched-to virtual purity-mitotically-competent oligodendrocyte progenitor cell pool, operationally separate and distinct from post-mitotic or mature oligodendrocytes (*Id.*).

Figures 1 and 2 of the '996 patent show his astrocyte/oligodendrocyte precursor cells differentiating directly to astrocytes and, to a much lesser extent, to oligodendrocytes with these mature cell types being characterized by various markers. It may be accurate to characterize rat oligodendrocytes and oligodendrocyte progenitors together at least with regard to their markers, because those markers are similar. Specifically, rat oligodendrocyte progenitors and oligodendrocytes both express the antigenic marker recognized by monoclonal antibody O4 (Third Goldman Declaration ¶ 7). In contrast, this marker is expressed by human oligodendrocytes and their immature forms, but NOT by mitotic oligodendrocyte progenitor cells (*Id.*). As a result, human oligodendrocyte progenitor cells cannot be acquired through the use of O4 as a selection marker, and O4-defined human oligodendroglial cells cannot act as mitotically-competent progenitor cells (*Id.*). This is in sharp distinction to the rat brain, in which the use of this marker can identify oligodendrocyte progenitors (*Id.*). The '996 patent does not recognize the non-applicability of this marker to the separation of human oligodendrocyte progenitor cells (*Id.*). In humans, mitotic cells biased strongly towards the oligodendrocyte lineage are instead recognized by the antigenic phenotype $O4^-$/PSA-$NCAM^-$/$A2B5^+$, which comprise a distinct subpopulation in which the CNP2 (i.e. cyclic nucleotide phosphodiesterase 2) promoter is transcriptionally activated (*Id.*).

As a result of these considerations, the selective propagation of mitotically-active oligodendrocyte progenitor cells from the rat brain does not predict the successful isolation of mitotic oligodendrocyte progenitor cells from postnatal or adult human brain tissue.

In view of the foregoing, the '996 patent cannot explicitly anticipate the claimed invention.

4. The '996 Patent Does Not Inherently Anticipate Claims 25, 26, and 29.

Having demonstrated that the '996 patent does not explicitly anticipate the invention of claims 25, 26, and 29, the only basis for a rejection under 35 U.S.C. § 102 is under the doctrine of inherency. During the March 15, 2005, personal interview, which is summarized in the Interview Summary mailed March 21, 2005, the examiner maintained that the '996 patent inherently discloses the claimed oligodendrocyte-specified and oligodendrocyte progenitor cells of claims 25, 26, and 29. Specifically, the examiner asserted that the '996 patent's multipotential oligodendrocyte-astrocyte precursor cells must inherently differentiate to the claimed oligodendrocyte-specified and oligodendrocyte progenitor cells before further differentiating to mature oligodendrocytes.

"Inherent anticipation requires that the missing descriptive material is 'necessarily present,' not merely probably or possibly present, in the prior art." *Trintec Indus., Inc. v. Top-U.S.A. Corp.*, 295 F.3d 1292, 1295, 63 USPQ2d 1597, 1599 (Fed. Cir. 2002) (quoting *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999)). In order for an element, not expressly disclosed in a prior art reference, to inherently anticipate, the missing element must be "necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991).

When a prior art rejection is based on the inherent characteristics of a claimed product, as disclosed in the cited art, the examiner's burden for maintaining the rejection, and appellants' burden in rebutting this rejection, is well-defined. "[W]hen the PTO shows sound basis for believing that the products of the applicant and the prior art are the same, the applicant has the burden of showing that they are not." *In re Spada,* 911 F.2d 705, 708, 15 USPQ2d 1655, 1658 (Fed. Cir. 1990). Evidence that "the PTO did not correctly apply or understand the subject matter of the reference, or [that] the PTO

drew unwarranted conclusions therefrom" will provide an adequate rebuttal. *Id.* The final rejection makes no effort to satisfy this standard.

Appellants have responded to the examiner's inherency position by submitting evidence in the form of the First Rao Declaration and the Second Rao Declaration which, as discussed *supra*, demonstrate that the astrocyte/oligodendrocyte precursor cells disclosed in the '996 patent are different from the oligodendrocyte-specified and oligodendrocyte progenitor cells claimed in the present application. In addition, the experimental work discussed in Example 15 of the '996 patent demonstrates that even when the inventors looked at the specific issue needed to support an inherency position (i.e. whether the mature oligodendrocytes and astrocytes produced were from a unipotential or multipotential A2B5+ cell population), they found that that population was multipotential.

Faced with all of this evidence from those working in the art, it was incumbent upon the examiner to present contrary evidence in the form of a declaration or otherwise. Manual of Patent Examining Procedure ("MPEP") § 2144.03 (2006). However, he did not do so. Instead, the examiner simply presented argument in disagreement with Dr. Rao. Given the fact that Dr. Rao is a co-inventor of the '996 patent and, therefore, clearly has superior knowledge of what cell types were and were not produced in the work described in the '996 patent, it is entirely inappropriate to disregard his testimony and, without any supporting evidence, reach a conclusion contrary to that of Dr. Rao. Further, even if the '996 patent could support a rejection based on inherency (which it cannot), there is no indication that it produces an enriched or purified preparation of oligodendrocyte-specified progenitor cells or oligodendrocyte progenitor cells.

In view of all the foregoing, it is apparent that there is no evidence to support a position that the '996 patent inherently anticipates the claimed invention.

5. The '996 Patent Does Not Render the Invention of Claims 25, 26, and 29 Obvious.

Having demonstrated that there is no basis for rejecting claims 25, 26, and 29 as anticipated by the '996 patent, the only prior art ground for rejection is obviousness under 35 U.S.C. § 103. However, this rejection also cannot stand.

In the final rejection, the examiner makes the following statement on the issue of obviousness:

> One of ordinary skill in the art at the time of filing would have been motivated to use the methods taught by Rao et al. [(i.e. the '996 patent)] to isolate an enriched or purified preparation of human mitotic oligodendrocyte progenitor cells from humans so that these pure cell preparations could be used to treat neurological disorders in humans, such as Parkinson's Disease, such as by transplantation of such cells into an afflicted individual. This motivation is suggested by Rao et al. and the reasonable expectation of success comes from the results of Rao et al. who successfully isolated such an enriched or purified preparation of mitotic oligodendrocyte progenitor cells from rat.

See Final Rejection, p. 4, lines 4-11. However, all that the '996 patent motivates those skilled in the art to do is find a preparation of bipotential oligodendrocyte-astrocyte precursor cells—not oligodendrocyte-specified progenitor cells, as claimed by appellants. As noted above, there is absolutely no suggestion in the '996 patent that there are oligodendrocyte-specified progenitor cells in rats (or any other species). Without that teaching, why would anyone skilled in the art be motivated to look for such a cell type? Not only is the suggestion needed to motivate one skilled in the art to look for the claimed cell type missing from the '996 patent, but that reference teaches away from even thinking about doing so. Having investigated the issue of whether the A2B5+ cells which generate oligodendrocytes and astrocytes in the '996 patent are multipotential or unipotential and having found (and taught) that they are multipotential, it is apparent that the '996 patent teaches away from the claimed invention. In view of this demotivating teaching, there is no basis for making an obviousness rejection. *See In re Fine,* 837 F.2d at 1074, 5 USPQ2d at 1599 ("[I]nstead of suggesting that the system be used to detect nitrogen compounds, Eads deliberately seeks to avoid them; it warns against rather than teaches Fine's invention.")

To the extent that the examiner's rejection based on the inherent teachings of the '996 patent is an obviousness rejection, it is particularly inappropriate. In this regard the following passage from *In re Rijckaert,* 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) is particularly instructive:

> 'The mere fact that a certain thing may result from a given set of circumstances is not sufficient [to establish inherency].' 'That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown.' Such a retrospective view of inherency is not a substitute for some teaching or suggestion supporting an obviousness rejection (citations omitted).

The examiner's inherency position clearly fails to comport with this standard.

Even if the examiner had established a *prima facie* case of obviousness, which he has not, that obviousness case would be rebutted by the objective evidence of non-obviousness of record in this case. *See Tec Air, Inc. v. Denso Mfg. Mich. Inc.,* 19 F.3d 1353, 1360 52 USPQ2d 1294, 1298 (Fed. Cir. 1999) ("[E]ven assuming that Denso established a prima facie case of obviousness, Tec Air presented sufficient objective evidence of nonobviousness to rebut it.").

The significance of appellants' present invention is apparent from the January 7, 2000, Research/Clinic Update for the National Multiple Sclerosis Society (attached hereto as Exhibit 12), which stated the following:

> Researchers at Cornell University Medical College, supported by the National MS Society, have for the first time isolated cells in the adult human brain that can divide and grow into myelin-making cells and that may ultimately be capable of replacing those damaged in multiple sclerosis.

The isolation of the adult human oligodendrocyte progenitor cell was thus chosen as one of the major MS-related discoveries of 1999 by the National Multiple Sclerosis Society. This work also merited a public affairs release of the Society for Neuroscience, which chose this discovery from thousands of annual research abstracts as one of its most important of the year, with an extensive and detailed release. A subsequent research summary by National MS Society (attached hereto as Exhibit 13) stated:

> Society-supported investigators at Cornell University Medical College reported, for the first time, being able to isolate immature ("progenitor") myelin-making cells in the adult human brain, remove them surgically and transform them, in laboratory dishes, into mature cells capable of making new myelin. This important step may provide a basis for new strategies for repairing damaged myelin in MS.

Appellants' work was reported in a number of both regional and national newspapers. An extensive report of appellants' work was reported in Newsday, then the largest circulation paper in New York City (attached hereto as Exhibit 14).

Both the significance and novelty of appellants' present invention are further apparent from its publication in the Journal of Neuroscience (first isolation of human oligodendrocyte progenitor cells: Roy et al., "Identification, Isolation and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter," *J. Neuroscience* 19:9986-95 (1999) (attached hereto as Exhibit 15), Journal of Neuroscience Research (first transplant of cells of human oligodendrocyte progenitor cells into demyelinated brain: Windrem et al., "Progenitor Cells Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Lesions of the Rat Brain," *J. Neurosci. Res.* 69:966-75 (2002) (attached hereto as Exhibit 16) (*cover photo*)); Nature Medicine (first transplant of human oligodendrocyte progenitor cells into prenatal brain: Nunes et al., "Identification and Isolation of Multipotential Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:439-447 (2003) (attached here to as Exhibit 17) (*cover photo*)); and again Nature Medicine (first transplantation of human oligodendrocyte progenitor cells into congenitally unmyelinated brain: Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Med.* 10:93-97 (2004) (attached hereto as Exhibit 18). These are among the pre-eminent journals in biomedicine. Nature Medicine currently has the highest impact factor of any journal in basic medical research, and its publication of work from the same laboratory twice in a year suggests the importance with which its editors view appellants' present invention and its uses. Thus, those skilled in art recognized that the present invention was a

substantial advance in the art over the '996 patent which did not report a means of isolating human oligodendrocyte-specified progenitor cells, let alone such progenitor cells themselves.

In the August 10, 2005, office action, the examiner characterized the pending claims as being akin to product-by-process claims where patentability must be established, in accordance with the MPEP § 2113, by the structure of the claimed product and not by the steps of making it except if those steps imply structure. Pursuant to MPEP § 2113, the examiner "bears a lesser burden of proof in making out a case of *prima facie* obviousness for product-by-process claims...than when a product is claimed in the conventional fashion." "Once the examiner provides a rationale tending to show that the claimed product appears to be the same or similar to that of the prior art, although produced by a different process, the burden shifts to applicant to come forward with evidence establishing an unobvious difference between the claimed product and the prior art product." MPEP § 2113.

Whether the analogy of the claimed invention to product-by-process claims is apt is questionable. Even more questionable is whether the examiner has met his burden of making a *prima facie* case of obviousness where there is no suggestion in the '996 patent to make the claimed oligodendrocyte-specified progenitor cells and, in fact, that reference has a teaching away from doing so. In any event, assuming that the present claims can be regarded as product-by-process claims, appellants have clearly met their responsibility to establish patentability over the '996 patent. In particular, to the extent the examiner has made a *prima facie* case of unpatentability, appellants have clearly presented evidence (i.e., the First and Second Rao Declarations) demonstrating otherwise. Simply put, the First and Second Rao Declarations as well as Example 15 of the '996 patent clearly demonstrate that the examiner's position that the claimed oligodendrocyte-specified progenitor cell is inherently present in or obvious from the '996 patent is wrong. Having shown this, the examiner must provide countervailing evidence (rather than mere argument or speculation). The examiner has provided no such evidence and, therefore, cannot properly maintain the rejection.

For all of these reasons, the obviousness rejection of claims 25, 26, and 29 based on the '996 patent cannot be maintained.

6. The Adult Human Cells of Claim 26 Are Further Distinguishable From the '996 Patent.

The mitotic oligodendrocyte progenitor cells from an adult human of claim 26 are further distinguishable from the astrocyte/oligodendrocyte precursor cells of the '996 patent. Differences in the method, time of isolation, and propagation suggest a difference between the cell types of claim 26 and those disclosed in the '996 patent (First Rao Declaration ¶ 7). The cells of claim 26 of the present application were derived from the adult brain using a promoter reporter based strategy where the CNP2 promoter directed expression of green fluorescent protein, whereas the astrocyte/oligodendrocyte precursor cells of the '996 patent were derived from fetal and neonatal tissue using cell surface antigen expression and fluorescence based antibody capture (*Id.*). No strategy of using CNP2 (a cytoplasmic marker) expression, a CNP2 promoter, or a related promoter reporter strategy is described in the '996 patent, nor is there any suggestion that such a promoter is transcriptionally active in all cells of the claimed enriched or purified preparation.

The '996 patent is directed to the enrichment of glial progenitor cells from newborn rat brain (First Rao Declaration ¶ 8). Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue (*Id.*). Yakovlev et al., "A Stochastic Model of Brain Cell Differentiation In Tissue Culture," *J. Math. Biol.* 37(1):49-60 (1998) (attached hereto as Exhibit 19); Bögler et al., "Measurement of Time In Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors is a Cellular Process Distinct from Differentiation or Division," *Dev. Biol.* 162(2):525-38 (1994) (attached hereto as Exhibit 20); and Raff et al., "Platelet-derived Growth Factor From Astrocytes Drives the Clock That Times Oligodendrocyte Development In Culture," *Nature* 333:562-65 (1988) (attached hereto as Exhibit 21) describe cell cycle changes as glial progenitor cells mature (*Id.*). They showed that adult cells differ in their cell cycle time and the number of divisions before they will become postmitotic (*Id.*). The present patent application discloses this for adult human-derived cells (*Id.*). In addition, adult-derived human oligodendrocyte progenitor cells differentiate as oligodendrocytes and produce myelin much more quickly than do

fetal or neonatal oligodendrocyte progenitor cells (*Id.*). In particular, as recently reported in Nunes et al., "Identification and Isolation of Multipotential Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:439-447 (2003) (attached hereto as Exhibit 17) and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004) (attached hereto as Exhibit 18), adult-derived oligodendrocyte progenitor cells not only myelinate much more rapidly than do fetal oligodendrocyte progenitors, but they do so more efficiently, with a higher proportion exhibiting effective myelin production, and myelinating a greater number of neuronal axons per donor cell than their fetal-derived counterparts (*Id.*). Adult cells are thus fundamentally more biased towards generating oligodendrocytes, towards maturing to express myelin proteins, and towards myelinating host axons (*Id.*). Moreover, adult cells execute all of these functions and achieve each of these cellular milestones much more quickly than fetal cells (*Id.*). As a result, they lend themselves to a very different set of potential clinical targets than fetal or neonatal-derived progenitors, as recently reported in Roy et al., "Progenitor Cells of the Adult Human Subcortical White Matter," In: *Myelin Biology and Disorders*, Vol. 1. R. Lazzarini, ed. Elsevier:Amsterdam, pp. 259-287 (2004) (attached hereto as Exhibit 22) (*Id.*).

Accordingly, adult oligodendrocyte progenitor cells are fundamentally different from fetal or neonatal-derived progenitors and, therefore, the '996 patent's rat fetal astrocyte/oligodendrocyte precursor cells are very different from the adult oligodendrocyte progenitor cells in claim 26 of the present application (*Id.*).

VIII. CONCLUSION

In view of the foregoing, it is clear that the rejection of the claims under 35 U.S.C. §§ 102(e) and 103(a) cannot be sustained. Accordingly, the final rejections should be reversed.

Dated: August 14, 2006

Respectfully submitted,

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Certificate of Mailing - 37 CFR 1.8(a)	
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450, on the date below.	
8/14/06	Laura L. Trost
Date	Laura L. Trost

IX. CLAIMS APPENDIX

25. An enriched or purified preparation of human mitotic oligodendrocyte-specified progenitor cells, wherein the mitotic oligodendrocyte-specified progenitor cells are from a post-natal human and a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of the enriched or purified preparation.

26. An enriched or purified preparation of human mitotic oligodendrocyte progenitor cells, wherein the mitotic oligodendroctye progenitor cells are from an adult human and a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of the enriched or purified preparation.

29. An enriched or purified preparation of human mitotic oligodendrocyte-specified progenitor cells, wherein a human cyclic nucleotide phosphodiesterase 2 promoter is transcriptionally active in all cells of the enriched or purified preparation.

X. EVIDENCE APPENDIX

A. **EXHIBIT 1** – Declaration of Mahendra S. Rao, M.D., Ph.D. Under 37 C.F.R. § 1.132

- Introduced into the record by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

B. **EXHIBIT 2** – Second Declaration of Mahendra S. Rao, M.D., Ph.D. Under 37 C.F.R. § 1.132

- Introduced into the record by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

C. **EXHIBIT 3** – Rao et al., "Glial-Restricted Precursors are Derived from Multipotent Neuroepithelial Stem Cells," *Devel. Biol.* 188:48-63 (1997)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

D. **EXHIBIT 4** – Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002)

- Introduced by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

E. **EXHIBIT 5** – Vallstedt et al., "Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain," *Neuron* 45:55-67 (2005)

- Introduced by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

F. **EXHIBIT 6** – Cai et al., "Generation of Oligodendrocyte Precursor Cells From Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005)

- Introduced by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

G. **EXHIBIT 7** – Pringle et al., "*Fgfr3* Expression by Astrocytes and Their Precursors: Evidence That Astrocytes and Oligodendrocytes Originate in Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003)

- Introduced by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

H. **EXHIBIT 8** – Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences In GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002)

- Introduced by appellant on May 25, 2005, and considered by the examiner in the office action, dated August 10, 2005.

I. **EXHIBIT 9** – Declaration of Steven A. Goldman under 37 C.F.R. § 1.132 ("First Goldman Declaration")

- Introduced by appellant on June 4, 2001, and considered by the examiner in the office action, dated August 28, 2001.

J. **EXHIBIT 10** – Third Declaration of Steven A. Goldman Under 37 C.F.R. § 1.132

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

K. **EXHIBIT 11** – Kirschenbaum et al., "*In Vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Forebrain," *Cerebral Cortex* 6:576-89 (1994)

- Introduced by the PTO in the December 5, 2000, office action, to which appellant responded on June 4, 2001. Also presented by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

L. **EXHIBIT 12** – January 7, 2000, Research/Clinic Update for the National Multiple Sclerosis Society

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

M. **EXHIBIT 13** – Summary of MS Research Progress – 1999, National MS Society, December 10, 1999

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

N. **EXHIBIT 14** – "Beyond the Gray Area," Newsday Article, Jamie Talan

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

O. **EXHIBIT 15** – Roy et al., "Identification, Isolation and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter," *J. Neuroscience* 19(22):9986-9995 (1999)

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

P. **EXHIBIT 16** – Windrem et al., "Progenitor Cells Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Lesions of the Rat Brain," *J. Neurosci. Res.* 69:966-75 (2002)

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

Q. **EXHIBIT 17** – Nunes et al., "Identification and Isolation of Multipotential Neural Progenitor Cells From the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:439-447 (2003)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

R. **EXHIBIT 18** - Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

S. **EXHIBIT 19** – Yakovlev et al., "A Stochastic Model of Brain Cell Differentiation In Tissue Culture," *J. Math. Biol.* 37(1):49-60 (1998)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

T. **EXHIBIT 20** – Bögler et al., "Measurement of Time In Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors Is a Cellular Process Distinct from Differentiation or Division," *Dev. Biol.* 162(2):525-38 (1994)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

U. **EXHIBIT 21** – Raff et al., "Platelet-derived Growth Factor from Astrocytes Drives the Clock that Times Oligodendrocyte Development In Culture," *Nature* 333:562-65 (1988)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

V. **EXHIBIT 22** – Roy et al., "Progenitor Cells of the Adult Human Subcortical White Matter," In: *Myelin Biology and Disorders*, Vol. 1. R. Lazzarini, ed. Elsevier:Amsterdam, pp. 259-287 (2004)

- Introduced by appellant on October 21, 2004, and considered by the examiner in the office action, dated January 13, 2005.

W. **EXHIBIT 23** – Noble et al., "The O2A (Adult) Progenitor Cell: A Glial Stem Cell of the Adult Central Nervous System," *Seminars in Cell Biol.* 3:413-22 (1992)

- Introduced by appellant on December 18, 2003, and considered by the examiner in the office action, dated March 24, 2004.

XI. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board on related appeals or interferences.

PATENT
Docket No.: 19603/4230 (CRF D-2238B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	8339	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

DECLARATION OF MAHENDRA S. RAO, M.D., PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, MAHENDRA S. RAO, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132, declare:

1. I received an M.D. (MBBS) degree in Medicine from Bombay University, Bombay India and a Ph.D. degree in Medicine from California Institute of Technology in Pasadena, California.

2. I am a Section Chief for the Stem Cell Unit at the Laboratory of Neuroscience, at NIA (National Institute on Aging), Triad Technology Center, 333 Cassell Drive, Baltimore, MD 21224; an Associate Professor of Neurosciences at Johns Hopkins University School of Medicine, Baltimore, MD 21224; and an Associate Professor at NCBS, Bangalore, India.

3. I am a founder of and shareholder in Q Therapeutics, Inc., 615 Arapeen Drive, Suite 102, Salt Lake City, Utah 84108, which I understand has an exclusive license under the present patent application.

4. I am familiar with the subject matter of the present patent application which I understand is directed to an enriched or purified preparation of human mitotic oligodendrocyte progenitor cells where the cyclic nucleotide phosphodiesterase 2 promoter (i.e. CNP2") is transcriptionally active in all cells of the enriched or purified preparation.

5. I am a co-inventor of U.S. Patent No. 6,361,996 ("'996 Patent"), which I understand has been used as a basis for rejecting claims in the above application. I present this declaration to demonstrate why the subject matter of the '996 Patent is very different from that of the present patent application.

6. The '996 Patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells. The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons. The '996 Patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added)(column 23, lines 1-5). Similarly, my paper Rao, et. al., "Glial-Restricted Precursors are Derived From Multipotential Neuroepithelial Stem Cells," *Devel. Biol.* 188: 48-63 (1997) clearly demonstrates that such A2B5+/NCAM cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage. The '996 Patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells of the present patent application and, therefore, are very different from the cells described in this present application.

7. Differences in the method, time of isolation, and propagation should also be noted. The cells in the present application were derived from the adult brain using a promoter reporter based strategy where the CNP2 promoter directed expression of green fluorescent protein. On the other hand, the astrocyte/oligodendrocyte precursor cells of the '996 Patent were derived from fetal and neonatal tissue using cell surface antigen expression and fluorescence based antibody capture. No strategy of using CNP2 (a cyctoplasmic marker) expression, a CNP2 promoter, or a related promoter reporter strategy is described in the '996 Patent.

8. The '996 Patent is directed to the enrichment of glial progenitor cells from newborn rat brain. Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue. Yakovlev, et. al., "A Stochastic Model of Brain Cell Differentiation in Tissue Culture," *J Math Biol.*, 37(1):49-60 (1998)(Appendix 1); Bogler et. al., "Measurement of Time in Oligodendrocyte-type-2

Astrocyte (O-2A) Progenitors is a Cellular Process Distinct from Differentiation or Division," *Dev Biol.*, 162(2):525-38 (1994)(Appendix 2); Raff et. al., "Platelet-derived Growth Factor From Astrocytes Drives the Clock That Times Oligodendrocyte Development in Culture." *Nature* 333(6173):562-65 (1988)(Appendix 3) describe cell cycle changes as glial progenitor cells mature. They showed that adult cells differ in their cell cycle time and the number of divisions before they will become postmitotic. The present patent application discloses this for adult human-derived cells. In addition, adult-derived human oligodendrocyte progenitor cells differentiate as oligodendrocytes and produce myelin much more quickly than do fetal or neonatal oligodendrocyte progenitor cells. In particular, as recently reported in Nunes et al., "Identification and Isolation of Multipotent Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Medicine* 9:239-247 (2003) (Appendix 4) and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain," *Nature Medicine* 10:93-97 (2004) (Appendix 5), adult-derived oligodendrocyte progenitor cells not only myelinate much more rapidly than do fetal oligodendrocyte progenitors, but they do so more efficiently, with a higher proportion exhibiting effective myelin production, and myelinating a greater number of neuronal axons per donor cell than their fetal-derived counterparts. Adult cells are thus fundamentally more biased towards generating oligodendrocytes, towards maturing to express myelin proteins, and towards myelinating host axons. Moreover, adult cells execute all of these functions, and achieve each of these cellular milestones, much more quickly than fetal cells. As a result, they lend themselves to a very different set of potential clinical targets than fetal or neonatal-derived progenitors, as recently reported in Roy et al., "Progenitor Cells of the Adult Human Subcortical White Matter In: *Myelin Biology and Disorders*, vol. 1. R. Lazzarini, ed. Elsevier:Amsterdam, pp. 259-287 (2004) (Appendix 6). The adult oligodendrocyte progenitor cells of the present application are thus fundamentally different from the fetal or neonatal-derived astrocyte/oligodendrocyte precursor cells of the '996 Patent.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/14/04

Mahendra S. Rao MD., Ph.D.

PATENT
Docket No.: 19603/4230 (CRF D-2238B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: R. Hutson
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Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

SECOND DECLARATION OF MAHENDRA S. RAO, M.D., PH.D.
UNDER 37 C.F.R. §1.132

Commissioner for Patents
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Dear Sir:

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progenitor cells where the cyclic nucleotide phosphodiesterase 2 promoter (i.e. CNP2) is transcriptionally active in all cells of the enriched or purified preparation.

5. I am a co-inventor of U.S. Patent No. 6,361,996 ("'996 Patent"), which I understand has been used as a basis for rejecting claims in the above application. I present this declaration to demonstrate why the subject matter of the '996 Patent is very different from that of the present patent application.

6. The '996 Patent discloses multipotential neuroepithelial stem cells and lineage-restricted astrocyte/oligodendrocyte precursor cells. The astrocyte/oligodendrocyte precursor cells are derived from neuroepithelial stem cells, are capable of self-renewal, and can differentiate into astrocytes and oligodendrocytes but not neurons. The '996 Patent characterizes these cells as "multipotential intermediate precursor cells restricted to glial lineages" (emphasis added)(column 23, lines 1-5). Similarly, my paper Rao, et al., "Glial-Restricted Precursors are Derived From Multipotential Neuroepithelial Stem Cells," *Devel. Biol.* 188: 48-63 (1997) clearly demonstrates that such A2B5+/NCAM- cells are capable of generating both astrocytes and oligodendrocytes and do not appear committed to the oligodendrocyte lineage. The '996 Patent's astrocyte/oligodendrocyte precursor cells are in a less differentiated state than the oligodendrocyte progenitor cells of the present patent application and, therefore, are very different from the cells described in this present application.

7. As shown in Figures 1-2 of the '996 Patent, the astrocyte/oligodendrocyte precursor cells 14 and 54, respectively, differentiate directly into two cell types – i.e. of astrocytes and oligodendrocytes. We also know from clonal analysis that there is a homogeneous population of astrocyte/oligodendrocyte precursor cells in which individual cells generate oligodendrocytes and two kinds of astrocytes by the process described in the '996 Patent. It is important to note that multiple pathways to generate post-mitotic, mature oligodendrocytes, have been described. Anderson and colleagues have shown that an oligodendrocyte/motorneuron precursor exists that does not make astrocytes (Zhou et al., "The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification," *Cell* 109:61-73 (2002) (attached hereto as Exhibit 1)). Other investigators have shown distinct sites of origin of oligodendrocytes and astrocytes presumably from separate precursors (Vallstedt et al., "Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain," *Neuron* 45:55-67 (2005) (attached hereto as Exhibit 2) and Cai et al., "Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005) (attached hereto as Exhibit 3)). Yet other investigators have shown that different kinds of oligodendrocyte progenitors exist (Pringle et al., "*Fgfr3* Expression by Astrocytes and Their

Precursors: Evidence that Astrocytes and Oligodendrocytes Originate in Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003) (attached hereto as Exhibit 4)). We are not aware of any evidence that the astrocyte/oligodendrocyte precursor cells of the '996 Patent generated mature oligodendrocytes by way of an intermediate oligodendrocyte-specific precursor. Indeed, Gregori et al., "The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal-Ventral Differences in GRP Cell Function," *J. Neurosci.* 22(1):248-256 (2002) (attached hereto as Exhibit 5) have suggested that the '996 patent describes a glial progenitor that gives rise to a more restricted astrocyte/oligodendrocyte precursor that still directly makes predominantly astrocytes and a small minority of oligodendrocytes. Thus, cells in the '996 Patent's pathway to oligodendrocyte production are bi-potential astrocyte/oligodendrocyte progenitor cells that have strong astrocytic bias. These cell types are very different from the oligodendrocyte-specified progenitor cells of the present application.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 05/10/05

Mahendra S. Rao MD., Ph.D.

DEVELOPMENTAL BIOLOGY 188, 48–63 (1997)
ARTICLE NO. DB978597

Glial-Restricted Precursors Are Derived from Multipotent Neuroepithelial Stem Cells[1]

M. S. Rao* and Margot Mayer-Proschel†,[2]

**Department of Neurobiology and Anatomy, University of Utah Medical School, 50 North Medical Drive, Salt Lake City, Utah 84132; and †Huntsman Cancer Institute, University of Utah, Biomedical Polymers Research Building 5701, Room 410C, Salt Lake City, Utah 84112*

Neuroepithelial cells in the developing ventricular zone differentiate into neurons, astrocytes, and oligodendrocytes. It is not known, however, whether this differentiation occurs in a single step or is a pathway utilizing intermediate more restricted precursor cells. To characterize the generation of glial cells from multipotent stem cells we have cultured neuroepithelial (NEP) cells from E10.5 rat embryos. Cultured NEP cells do not express any glial differentiation markers when grown on fibronectin/laminin under nondifferentiation conditions. NEP cells, however, differentiate into A2B5 immunoreactive cells which can subsequently give rise to oligodendrocytes and astrocytes. Clonal analysis of NEP cells demonstrates that the A2B5 immunoreactive cells arise in clones that contain neurons and astrocytes, indicating that A2B5$^+$ cells arise from multipotent NEP precursor cells. A2B5$^-$ cells, maintained as undifferentiated cells over multiple passages, can subsequently give rise to both oligodendrocytes and astrocytes. A2B5$^+$ cells, however, do not generate neurons. Thus A2B5$^-$ cells represent a restricted progenitor cell population that differentiates from a multipotent NEP cell. Based on our results we propose that differentiation of the multipotential NEP cells to terminally differentiated glial cells occurs via intermediate restricted precursors. © 1997 Academic Press

INTRODUCTION

Multipotent cells with the characteristics of stem cells have been identified in several regions of the central nervous system and at several developmental stages (for review see Gage *et al.*, 1995; Marvin and McKay, 1992; Skoff, 1996). These cells, often referred to as neuroepithelial stem cells (NEP cells), have the capacity to undergo self renewal and to differentiate into neurons, oligodendrocytes, and astrocytes (Davis and Temple, 1994; Gritti *et al.*, 1996; Reynolds *et al.*, 1992; Reynolds and Weiss, 1996; Williams *et al.*, 1991). The nervous system also contains precursor cells with restricted differentiation potentials (Kilpatrick *et al.*, 1995; Price *et al.*, 1987, 1991; Reynolds *et al.*, 1992; Reynolds and Weiss, 1996; Williams, 1995; Williams *et al.*, 1991). Although it is likely that lineage-restricted cells are the progeny of multipotent cells, previous studies have not demonstrated a direct relationship between these two classes of cells (see reviews, Morrison *et al.*, 1997; Stemple and Mahanthappa, 1997; Temple and Qian, 1996).

To demonstrate a relationship between multipotent stem cells and more restricted precursors we analyzed the development of glial-restricted precursors from NEP cells. We chose to focus on the transition of multipotent NEP cells to glial precursor cells, as glial precursor cells are the best characterized intermediate precursor cells in the CNS. The most extensively studied precursor is called the oligodendrocyte-type-2 astrocyte progenitor (O-2A) (Noble *et al.*, 1990; Raff *et al.*, 1983; Tempel and Raff, 1986; Temple and Raff, 1985). O-2A precursors can be isolated and purified from postnatal rat optic nerve, cortex, and spinal cord and differentiate into postmitotic GalC$^-$ oligodendrocytes or into A2B5$^-$/GFAP$^-$ type-2 astrocytes, which are distinct from GFAP$^-$/A2B5$^-$ type-1 astrocytes (Raff *et al.*, 1983), depending on the culture conditions. O-2A progenitor cells exhibit a proliferate response to a variety of mitogens, such as platelet-derived growth factor (PDGF) and basic fibroblast

[1] This work was supported by the Huntsman Cancer Institute award to M.S.R. and M.M-P. and the Muscular Dystrophy Association and NIH first award to M.S.R. We thank Dr. Mark Noble for his enthusiastic interest in this work and helpful criticism, Tahmina Mujtaba and Samuel Bernard for excellent technical assistance, and Dr. Chris Proschel for his support through all phases of this project. We also thank all members of our laboratories for constant stimulating discussions.

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growth factor (bFGF) (Gard and Pfeiffer, 1990; Mayer *et al.*, 1993; McKinnon *et al.*, 1990; Noble *et al.*, 1988) and can be kept in an undifferentiated dividing stage as long as both PDGF and bFGF are present in the culture medium (Bogler *et al.*, 1990). We have used antigenic markers and culture conditions described in the O-2A system to determine glial generation in the early spinal cord.

NEP cells derived from the embryonic spinal cord can be maintained in an undifferentiated stage over several passages but retain the ability to generate neurons, oligodendrocyte, and astrocytes (Rao *et al.*, (1996) *Neuroscience* Abstract 215.12). We show that NEP cells differentiate into A2B5 immunoreactive cells that lack markers for other cell lineages and have a morphology similar to that of O-2A progenitor cells isolated from other embryonic brain regions (Aloisi *et al.*, 1992; Fok-Seang and Miller, 1994; Temple and Raff, 1985). We provide evidence that these A2B5$^+$ cells arise from multipotent NEP cells, can be expanded for multiple passages without losing their multipotentality, and can generate oligodendrocytes and astrocytes but not neurons. NEP-derived A2B5$^+$ cells thus represent glial-restricted intermediate precursor cells. Using this system, we are able to demonstrate for the first time that cells can progress from a multipotential stage to a terminal differentiated end stage via intermediate lineage-restricted precursor cells.

MATERIALS AND METHODS

Substrate Preparation

Laminin, used at a concentration of 20 μg/ml (Biomedical Technologies Inc.), was dissolved in distilled water and applied to tissue culture plates (Falcon). For the fibronectin solution, fibronectin (Sigma) was resuspended to a stock concentration of 10 mg/ml and stored at −80°C and diluted to a concentration of 250 μg/ml in DPBS (Gibco). The fibronectin solution was applied to tissue culture dishes and immediately withdrawn. Subsequently the laminin solution was applied and plates were incubated for 5 hr. Excess laminin was withdrawn and the plates were allowed to air dry. Plates were rinsed with water and then allowed to dry again.

Neuroepithelial Cell Cultures

Sprague–Dawley rat embryos were removed at Embryonic Day 10.5 (13–22 somites) and placed in a petri dish containing Hanks balanced salt solutions (HBSS, Gibco). The trunk segments of the embryos (last 10 somites) were dissected using tungsten needles, rinsed, and then transferred to fresh HBSS. Trunk segments were incubated at 4°C in 1% trypsin solution (Gibco) for a period of 10 to 12 min. The trypsin solution was replaced with fresh HBSS containing 10% fetal bovine serum (FBS). The segments were gently triturated with a Pasteur pipette to release neural tubes free from surrounding somites and connective tissue. Isolated neural tubes were transferred to a 0.05% trypsin/EDTA solution (Gibco) and incubated for 10 min. Cells were dissociated by trituration and plated in 35-mm dishes (Nunc) at high density. Cells were maintained at 37°C in 5% CO_2/95% air. Cells were replated at low density after 3 days. Cells from several dishes were then harvested by trypsinization (0.05% Trypsin/EDTA solution for 2 min), pelleted, resuspended in a small volume, and replated at a density of 5000 cells/35-mm dish. The basal medium used in all experiments was a chemically defined medium modified from that described by Stemple *et al.* (1988). The medium consisted of DMEM-F12 (Gibco) supplemented with additives described by Bottenstein and Sato (1979) and bFGF (20 ng/ml) and CEE extract (10%) prepared as described previously (Stemple and Anderson, 1992).

Clonal Cultures of Neuroepithelial Cells

Cells were trypsinized and plated in 35-mm dishes coated with fibronectin/laminin at a dilution of 50 cells/dish. In some experiments cells were plated at 10 cells/dish. Cells were allowed to settle for a period of 4 hr, single cells were circled, and their development was followed in culture. In most experiments clonal cultures were terminated after 12 days. In experiments to demonstrate oligodendrocyte development clones were followed for 18–21 days. In these assays approximately 20–40% of single cells died within 24 hr. Of the remaining cells (60–80%) the large majority (< 90%) generated multipotent clones. Clonal plates were usually triple-labeled with the cell surface antigen and the appropriate secondary antibodies.

Generation of Neurons, Oligodendrocytes, and Astrocytes

Neuroepithelial cells cultured in nondifferentiating conditions for a period of 5 days were harvested by trypsinization and replated onto dishes sequentially coated with fibronectin/laminin (0.25 mg/ml) in neuroepithelial culture medium. For neuronal and oligodendrocyte differentiation, the medium consisted of neuroepithelial culture medium described above with the omission of 10% CEE (-CEE condition). For astrocyte differentiation the -CEE medium was supplemented with 10% fetal calf serum. Differentiation was assayed 5 days or 9 days after replating (as detailed in the results).

Immunopanning of A2B5$^+$ Cells

NEP cells were cultured in -CEE conditions for 6 days and the A2B5$^+$ cell population was purified using a specific antibody-capture assay (Wysocki and Sato, 1978) with modification utilized previously (Mayer *et al.*, 1994). In brief, cells were trypsinized and the suspension was plated on an A2B5 antibody (Eisenbarth *et al.*, 1979)-coated dish to allow binding of all A2B5$^+$ cells to the plate. The supernatant was removed and the plate was washed with DMEM supplemented with additives described by Bottenstein and Sato (1979) (DMEM-BS). The bound cells were scraped off and plated on fibronectin/laminin-coated glass coverslips in 300 μl DMEM-BS ± growth factors at 5000 cells/well. In the final culture the contaminating number of A2B5$^-$ cells represented less than 10% of the total cells. The A2B5 antibody for coating the plates was used at a concentration of 5 μg/ml protein. Cells were allowed to bind to the plate for 20–30 min in a 37°C incubator. Growth factors were added every other day at a concentration of 10 ng/ml. Recombinant human PDGF-AA was a kind gift from C. George-Nascimento and L. Coussens (Chiron Corp.). Recombinant rat CNTF was obtained from Precision Research Biochemicals. Recombinant bFGF was purchased from PeroTech Inc. and retinoic acid (RA) was obtained from Sigma.

Clonal Cultures of A2B5⁺ Cells

After CEE withdrawal cells were stained with A2B5 and IgM-monoclonal antibody FITC (Southern Biotechnologies) and plated at a limited dilution in 96-well plates coated with fibronectin/laminin at a dilution of 1 cell/well. Cells were allowed to settle for a period of 4 hr and wells containing one A2B5⁺ stained cell were recorded. Cells were cultured in the presence of PDGF and bFGF for 7 days in which clones usually reached a size of 50–200 cells/clone. After washing with bFGF-free DMEM-BS individual clones were grown in a medium supplemented with PDGF. In most experiments clonal cultures were stained after 12 days. In experiments designed to demonstrate oligodendrocyte development clones were followed for 18–21 days. In experiments where self-renewal capacity was demonstrated, clones were replated into 35-mm dishes and further expanded with PDGF/bFGF. Clones were propagated through four passages. After each passage clones were stained with A2B5 to determine their homogeneity.

Immunocytochemistry

Staining procedures were as described previously (Mayer *et al.*, 1994). The antibodies used are listed in Table 1. Staining for the cell surface markers A2B5, α-GalC, O4 (cell lines obtained from ATCC), and p75 (Yokoyama *et al.*, 1993) was carried out in cultures of living cells. To stain cells with antibodies against internal antigens like GFAP (Sigma), which recognizes specifically astrocytes (Bignami *et al.*, 1972), β-III tubulin (DAKO) and RT-97, which stain neurons (Geisert and Frankfurter, 1989), nestin, a marker for undifferentiated stem cells (Lendahl *et al.*, 1990), or 5-bromodeoxyuridine (BrdU, Sigma), to determine the number of dividing cells, cultures were fixed with ice-cold methanol. All secondary monoclonal antibodies were purchased from Southern Biotechnology.

Double labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies followed by non-cross-reactive secondary antibodies. In triple label experiments, cultures were incubated with the primary antibody in blocking buffer for a period of 1 hr, rinsed with buffer (PBS), and incubated with a species-specific secondary antibody in blocking buffer for 1 hr. Cultures were rinsed three times with PBS and examined under a fluorescent microscope. For labeling cultures with four antibodies simultaneously, live cells were first incubated with the surface antibodies A2B5 and α-GalC without the secondary layers. Clones were then fixed in ice-cold methanol for 10 min and stained with α-β-III tubulin and the appropriate secondary antibody. After scoring the result of this staining, which one of it usually being negative, clones were stained with GFAP and the secondary layer for the first set of surface antibodies. Finally, the secondary antibody for GFAP was added. This procedure allowed staining with four antibodies using only three fluorescent-color conjugated secondary antibodies.

RESULTS

Neuroepithelial Cells Do Not Express Oligodendrocyte Lineage Markers in Vivo and in Vitro

Neurons, oligodendrocyte, and astrocytes can be identified using a variety of lineage-specific markers (Table 1) (Eisenbarth *et al.*, 1979; Geisert and Frankfurter, 1989; Sommer and Schachner, 1981; Trimmer *et al.*, 1991). In addition to defining differentiated cells, some precursor cells can also be recognized by specific antibodies. We employed two such markers in this study: nestin is expressed by a variety of undifferentiated cells in the CNS (Dahlstrand *et al.*, 1992a, 1992b; Lendahl *et al.*, 1990; Tohyama *et al.*, 1992, 1993; Zimmerman *et al.*, 1994), while the A2B5 antibody labels O-2A progenitor cells (for summary see Table 1).

NEP cells *in vivo* do not label with any lineage markers tested (Fig. 1, α-GalC and α-β-III tubulin not shown) as determined by labeling the neural tube sections of rat embryos at 10.5 days gestation with differentiation markers (Fig. 1 and Table 1). Nestin was, however, expressed uniformly throughout the tissue.

To determine whether NEP cells isolated from the neural tube at that stage remained negative for differentiation markers *in vitro*, dissociated NEP cells from the rat spinal cord at Day 10.5 were plated on fibronectin/laminin coated dishes. Cells were cultured in the presence of bFGF (10 ng/ml) and 10% chick extract (CEE) for 3 days. In this culture condition, cells grew as a population of undifferentiated precursor cells which can be propagated in culture for at least 5 passages as long as bFGF and CEE are constantly supplied (Rao *et al.* (1996) *Neuroscience* Abstract 215.12). NEP cell cultures were established and stained after 3 days of *in vitro* growth with antibodies present on lineage committed glial cells (Table 1). Consistent with the *in vivo* staining pattern, cultures were α-nestin⁺ and α-GFAP⁻ and A2B5⁻ (Fig. 2) as well as negative for other lineage markers (Table 1).

A2B5 Immunoreactivity Defines Glial-Specific Cells in the NEP Cell Population

A2B5 immunoreactivity identifies a glial precursor cell at various stages of development in the brain (Noll and Miller, 1993; Pringle and Richardson, 1993; Raff *et al.*, 1983; Warf *et al.*, 1991; Yu *et al.*, 1994) and spinal cord (Fok-Seang and Miller, 1994). We therefore examined the expression of A2B5 in NEP cultures. Seventy percent of NEP cells cultured in the absence of CEE for 3 days exhibit A2B5 immunoreactivity (data not shown, see, however, Fig. 3). These A2B5⁺ cells had a flat morphology and were able to divide in the presence of bFGF. After 4 days in culture in the absence of CEE, 81 ± 7% of A2B5⁺ NEP cells, stained with anti-BrDU for 24 hr, were engaged in cell division (Fig. 3). Double labeling the NEP-derived A2B5⁺ cells with the antibodies α-nestin, α-GalC, α-GFAP, α-β-III tubulin, and α-p75 (p75, an antibody against the low-affinity NGF receptor, has been described to recognize a subset of astrocytes) showed that none of the lineage markers were coexpressed on A2B5⁻ cells (Fig. 3). A substantial subset of the A2B5⁺ cells, however, expressed α-nestin. This coexpression of α-nestin and A2B5 has previously been described on O-2A progenitor cells. Thus NEP-derived A2B5⁺ cells are antigenically similar to O-2A progenitor cells.

A2B5⁺ cells when stained after an additional 2 days in

TABLE 1
Antibodies Which Identify Specific Cell Types

Antibody/dilution			Antigen recognized	Cell type recognized
A2B5[a]	Mouse (IgM)	1:2	Gangliosides	Glial precursors
O4	Mouse (IgM)	1:2	Galactoside	Oligodendrocytes/precursors
α-GalC	Mouse (IgG)	1:2	Galactocerebroside	Oligodendrocytes
α-GFAP	Rabbit (IgG)	1:500	Glial fibrillary acid protein	Astrocytes
α-β-III tubulin	Mouse (IgG)	1:400	β-III tubulin	Neurons
RT-97	Mouse (IgG)	1:5	Neurofilament	Neurons

Note. Antibodies were used in combination for double or triple label experiments.
[a] A2B5 was originally identified as a neuronal marker (Eisenbarth, 1979). We and others have found that A2B5 is specific for glial cells in this system.

cultures had begun to express glial-specific markers. A subpopulation of cells were clearly GalC$^+$ by that time. To confirm that cells were sequentially differentiating into oligodendrocytes, cultures were stained with O4 and α-GalC. As expected, 30% of the O4$^+$ cells coexpressed α-GalC, resembling immature oligodendrocytes. Double labeling with A2B5 and α-GFAP showed that 10% of the A2B5$^+$ cells were also GFAP$^+$, resembling the antigenic characteristic of type-2 astrocytes (Figs. 4A–4F). All the markers which were coexpressed at that later time point on a subset of A2B5$^+$ cells are characteristic of cells which have been described to belong to the O-2A lineage. This observation suggested that at least a subset of the A2B5$^+$ cells represented glial precursor cells and that A2B5 was a useful marker to define this subpopulation of cells in more detail.

A2B5$^+$ Cells Arise from Multipotential Stem Cells

To investigate whether A2B5$^+$ cells arise from multipotent NEP cells or whether A2B5$^+$ cells arise from an already committed subpopulation of A2B5$^-$ NEP cells, we plated cells at clonal densities and followed their development in culture for 10 days. Dishes were then double stained with the antibodies A2B5/α-β-III tubulin or A2B5/α-GFAP (Figs. 5A and 5B, respectively) and 132 clones were analyzed (Table 2). Nearly all 132 analyzed clones consisted of a mixture of A2B5$^+$, GFAP$^+$, and β-III tubulin$^+$ cells. Ninety-one percent of the clones contained cells that were either A2B5$^+$ or α-GFAP$^+$, while 93% of the clones were either A2B5$^+$ or α-β-III tubulin$^+$. None of the analyzed clones consisted only of cells which were A2B5$^-$. It is noteworthy that although at this early stage none of the clones contained GalC$^+$ cells, oligodendrocytes could be identified in clonal cultures and in mass cultures at later stages (12–15 days post-CEE condition).

The clonal analyses suggested that the A2B5$^+$ population arose from a common multipotential A2B5$^-$ precursor cell.

A2B5$^+$ Cells Differ from Neuroepithelial Cells in Their Differentiation Potential

The mass culture and the clonal experiments suggested that the marker A2B5$^+$ defines cells which might represent the precursors for glial cells but not for neurons. In order to determine directly whether NEP-derived A2B5$^+$ cells can only give rise to glial cells, we purified the A2B5$^+$ population by immunopanning (see Materials and Methods). After 5 days of culturing NEP cells in the absence of CEE, cells were immunopurified, plated on fibronectin/laminin-coated dishes, and exposed to cytokines previously associated with differentiation of precursor into oligodendrocytes, astrocytes, or neurons. The A2B5 panned population was >98% positive for A2B5$^+$ cells when stained 1 hr after panning. Staining 24 hr after plating showed that all cells of the panned population were A2B5$^+$ and did not express any other lineage markers tested (Fig. 6, α-β-III tubulin staining not shown).

Panned cultures, in the presence of bFGF alone for 5 days consisted of 1% oligodendrocytes, 50% GFAP$^+$ astrocytes, and 49% A2B5$^-$ cells. The proportion of differentiated cells was significantly shifted when the bFGF containing medium was replaced after 3 days with medium supplemented only with PDGF. In this condition, 30% of the culture consisted of oligodendrocytes, 50% of astrocytes, and 20% of the cells were A2B5$^+$ cells (Fig. 7).

Although growth in the presence of bFGF alone was sufficient to allow differentiation of NEP cells into neurons in the parent population (details of the parent population described in Kalyani *et al.*, 1997) we failed to detect any neurons in the A2B5$^+$-panned population cultured in the presence of bFGF (Fig. 7). To enhance the probability of neuronal differentiation, we additionally supplemented the medium with retinoic acid (in this culture condition 10 of 10 clones of the parent population contained β-III tubulin$^+$ neurons). Even in this neuron promoting environment the immunopurified A2B5$^+$ population did not contain β-III tubulin$^-$ cells. It was unlikely that we lost the neuronal population through selective cell death because we did not observe significant cell death in the panned mass cultures at any time, suggesting that neurons did not appear rapidly and died, nor did we detect any evidence of β-III tubulin$^-$ "ghosts."

These results suggested that the precursor cells which are responsible for generating neurons were not part of the

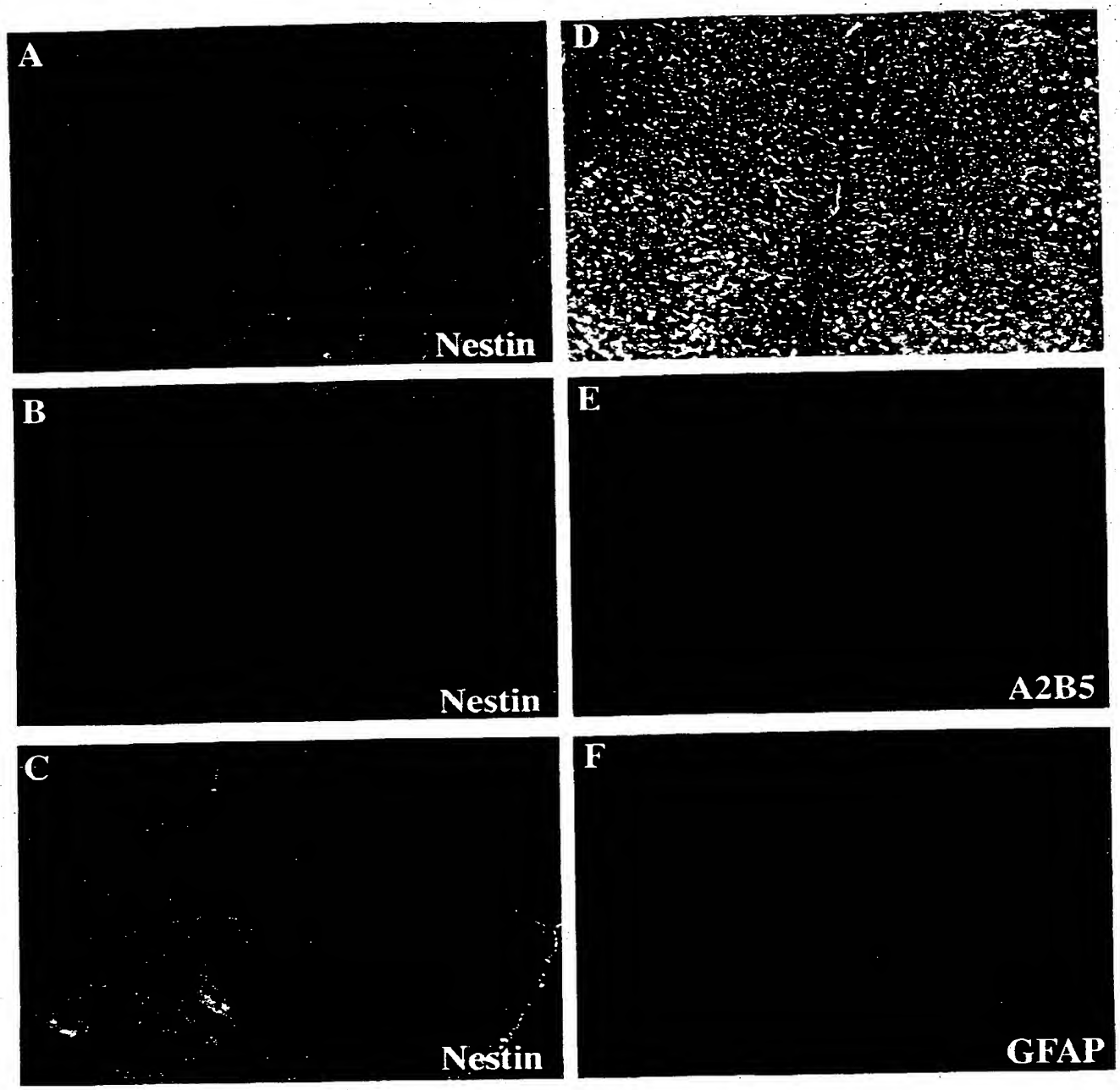

FIG. 1. Neural tube from rat embryos at 10.5 days gestation did not express glial lineage markers. E10.5 rat embryos were collected and neural tubes dissected, fixed, and sectioned. Sections were double labeled with α-nestin, α-GFAP, or A2B5 (A–F). A (fluorescence) and D (phase) show a representative section illustrating nestin expression in the neural tube. B, C, E, F show that nestin$^+$ cells are A2B5$^-$ and GFAP$^-$.

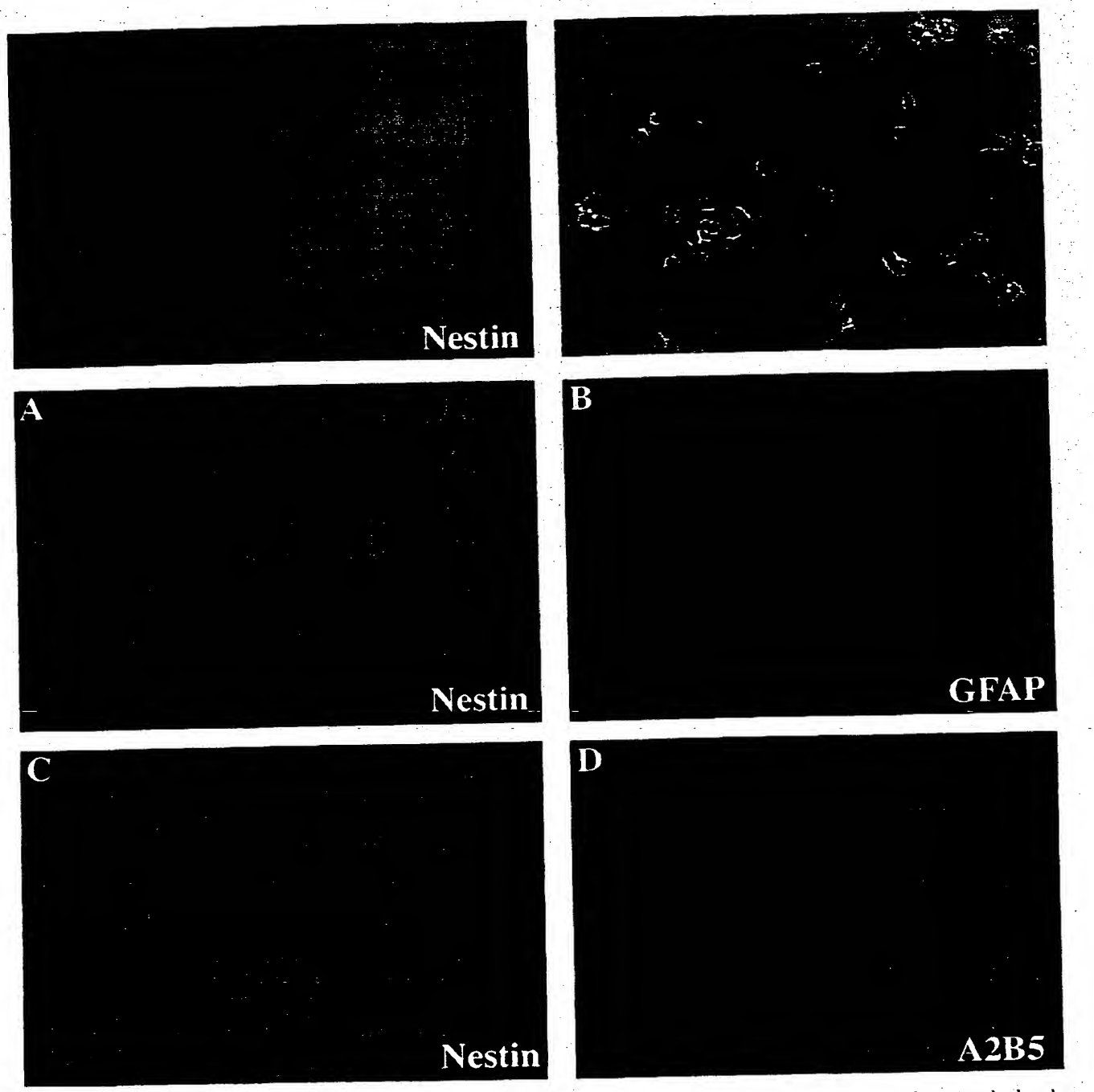

FIG. 2. NEP cells do not express glial markers *in vitro* in the presence of CEE. E10.5 rat neural tube cells were dissociated, plated at low density, and grown in the presence of bFGF and CEE for 5 days. Cells were double labeled with α-nestin and α-GFAP (A and B) or α-nestin and A2B5 (C and D). All NEP cells are nestin$^+$ and GFAP$^-$/A2B5$^-$.

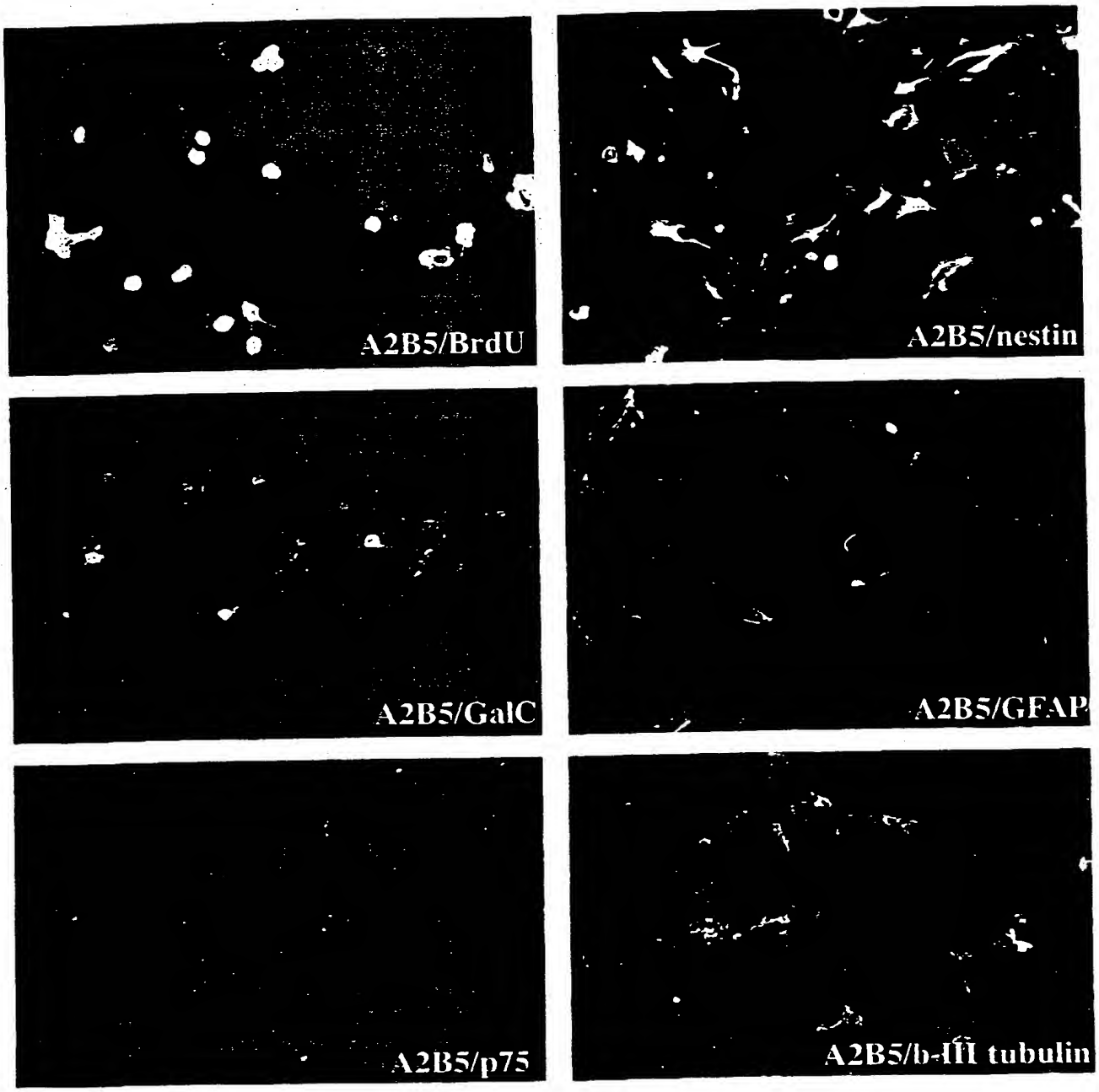

FIG. 3. A2B5$^+$ cells divide *in vitro* and do not express other differentiation markers after 5 days in culture. E10.5 rat neural tube cells were dissociated, grown for 3 days in the presence of CEE and bFGF, and then replated at 5000 cells/coverslip in medium devoid of CEE for an additional 5 days. Cells were incubated for 24 hr with BrDU and stained with anti-BrdU. Parallel cultures were double stained after 7 days with A2B5/α-nestin, A2B5/α-GalC, A2B5/α-GFAP, A2B5/α-p75, or A2B5/α-β-III tubulin. 20% of the A2B5$^+$ cells were nestin$^+$ while all A2B5$^+$ cells were negative for differentiation markers. A2B5 staining is shown in green (fluorescein) while α-BrDU, α-nestin, α-GalC, α-GFAP, α-p75, and α-β-III tubulin are stained in red (rhodamine).

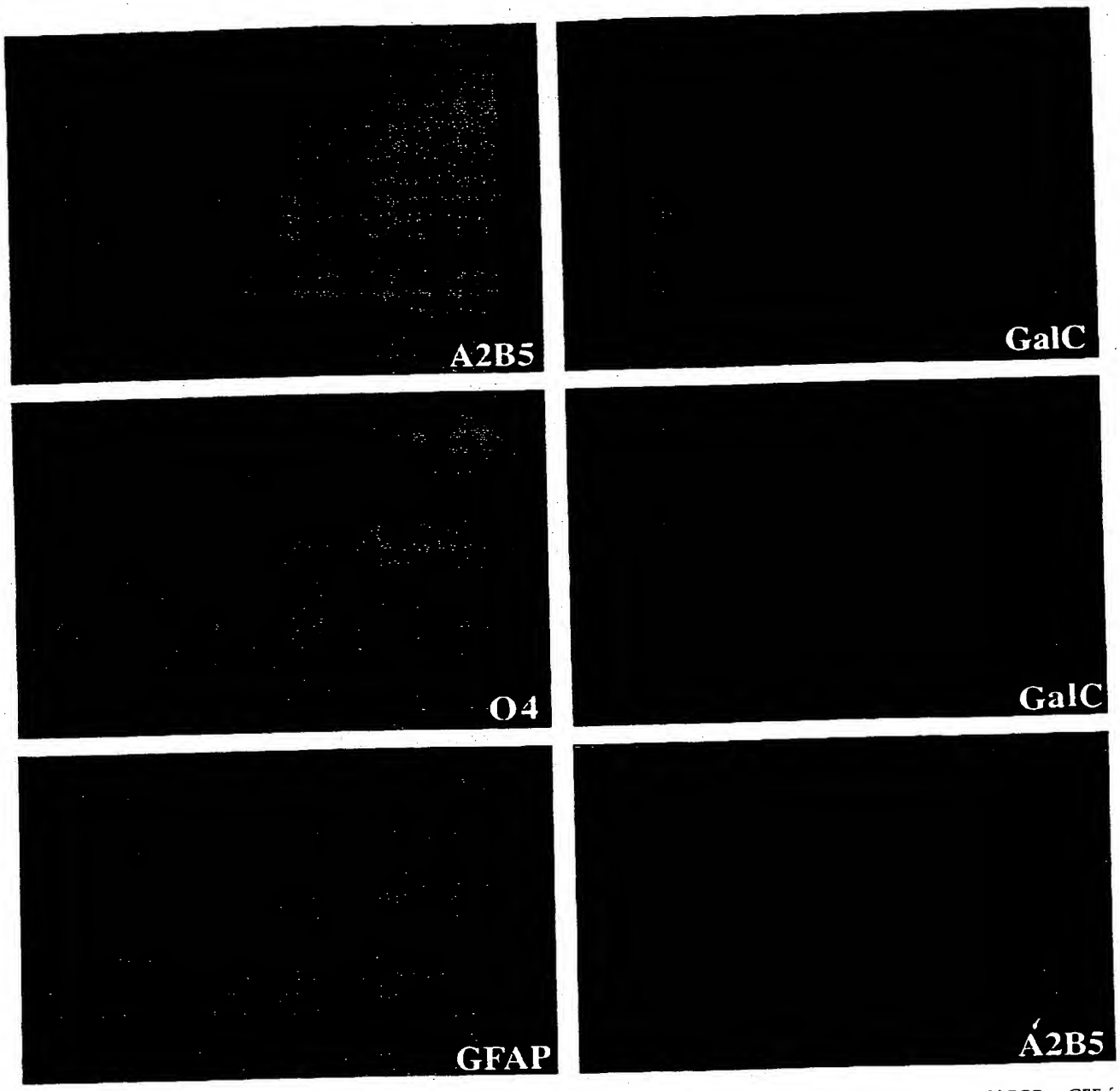

FIG. 4. A2B5 is coexpressed with other glial markers after 7 days in culture. E10.5 NEP cells grown in the presence of bFGF + CEE for 3 days were harvested, replated in bFGF containing NEP medium without additional CEE, and grown for an additional 7 days. Culture dishes were double labeled with A2B5/α-GalC, O4/α-GalC, or A2B5/α-GFAP. A subset of the A2B5$^+$ cells were also GalC$^+$ or GFAP$^-$. The GalC$^+$/O4+ cells represented immature oligodendrocytes (Bansal *et al.*, 1989; Gard and Pfeiffer, 1990; Sommer and Schachner, 1981; Warrington and Pfeiffer, 1992).

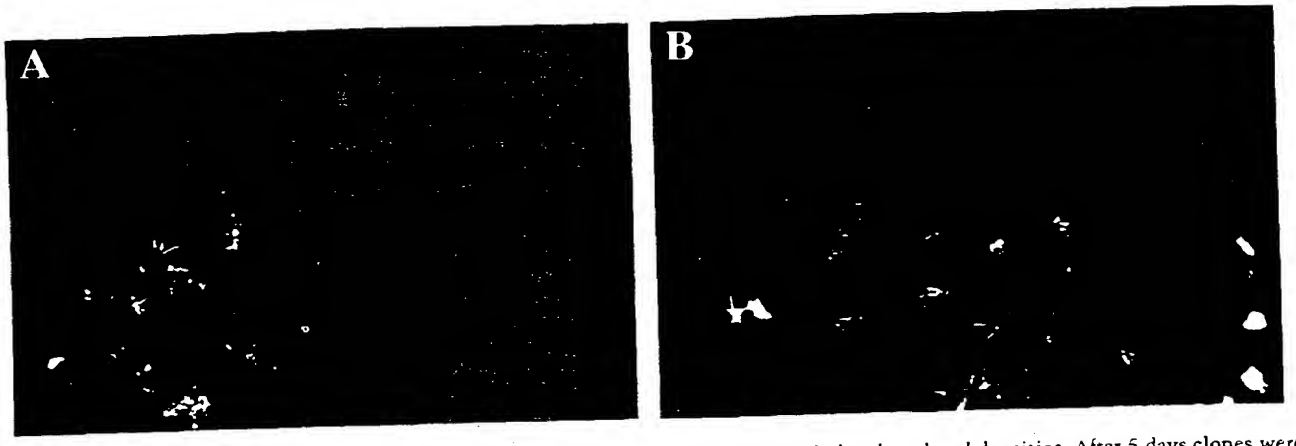

FIG. 5. A2B5$^+$ cells arise from multipotent NEP cells. NEP cells were dissociated and plated at clonal densities. After 5 days clones were stained with A2B5/α-β-III tubulin or A2B5/α-GFAP. Nearly all clones contained A2B5$^-$/β-III tubulin$^+$ cells (A) or A2B5$^+$/GFAP$^+$ cells (B). None of the clones consisted of solely A2B5$^+$ cells. A2B5 staining is shown in green (fluorescein), while α-β-III tubulin and α-GFAP are stained in red (rhodamine).

immunopurified A2B5$^+$ population. As the A2B5-panned cells gave rise to astrocytes and oligodendrocytes but not to neurons, it appeared that the A2B5$^+$ population contained precursor cells which were restricted to the glial lineage.

A2B5$^+$ Cells Generate Multipotential Glial Precursor Cells

Our mass culture experiments suggested that the A2B5 panned population contained cells with a differentiation potential restricted to glial lineages. This experiment, however, did not address whether astrocytes and oligodendrocytes are generated from committed unipotential cells present in the A2B5$^+$ population or whether single cells are bipotential and can generate both astrocytes and oligodendrocytes. To address this question we performed clonal experiments, wherein the A2B5-panned population was stained with A2B5 1 day after panning and cells were plated at limiting dilution in 96-well plates. Wells were scored with immunofluorescence and wells with one A2B5$^+$-stained cell were recorded and cultured in PDGF/bFGF for 7 days. This procedure allowed the expansion of clones and also minimized the amount of cell death occurring when single cells were plated directly into differentiation conditions (unpublished observation). After 7 days expanded clones contained from 50 to 200 cells and were uniformly A2B5$^+$.

TABLE 2
NEP Cells Generate Mixed Clones *in Vitro*

Antigen expressed	Percentage of clones	Number of clones
A2B5$^+$/β-III tubulin$^+$	93	71/76
A2B5$^-$/GFAP$^+$	91	51/56
A2B5$^+$ alone	0	0/132
Total number of clones analyzed	132	

Note. NEP cells were isolated and grown at clonal densities in the absence of CEE and in the presence of bFGF. After 7 days clones were stained with antibodies against A2B5/α-β-III tubulin or A2B5/α-GFAP. The majority of clones contained a mixture of cells either A2B5/β-III tubulin$^+$ or A2B5/GFAP$^+$. No pure clones were found.

The majority of the clones (51) were first washed with bFGF-free DMEM-BS and then switched to PDGF-supplemented medium, an effective culture condition to induce oligodendrocyte generation as shown in mass culture experiments. All clones contained oligodendrocytes, GFAP$^+$ astrocytes, and A2B5$^+$ cells, while none of the clones contained β-III tubulin$^+$ cells, suggesting that single A2B5$^+$ cells were at least bipotential and also were restricted to glial cell lineages (Table 3). A representative clone expanded in PDGF/bFGF, switched to PDGF, and stained after 7 days is shown in Fig. 8.

We also tested the differentiation potential of A2B5$^+$ cells in a culture medium supplemented with bFGF and CNTF. From our panned mass culture experiments, it seemed clear that bFGF alone leads to an increase in the number of GFAP$^+$ astrocytes and a decrease in the number of oligodendrocytes. Depending on culture conditions, CNTF has been shown to promote oligodendrocyte generation (Mayer *et al.*, 1994) or to lead to the generation of type-2 astrocytes, which express A2B5 and GFAP transiently (Lillien and Raff, 1990) or stably (Fok-Seang and Miller, 1992; Madarasz *et al.*, 1991). We analyzed a total of six clones, which were ex-

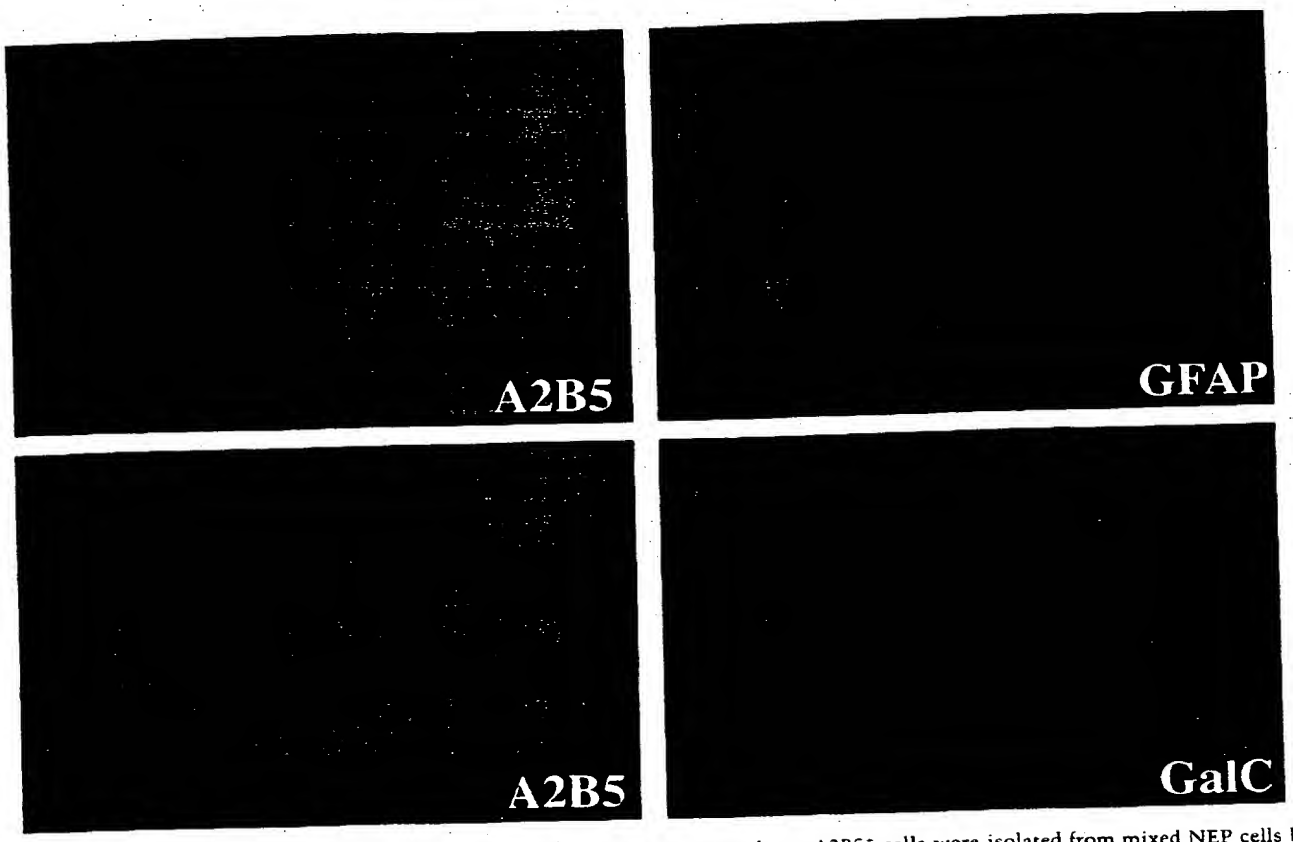

FIG. 6. Panned A2B5$^+$ cells are negative for lineage markers after 1 day is culture. A2B5$^+$ cells were isolated from mixed NEP cells by immunopurification and cultured in the presence of bFGF. Cells were stained after 24 hr with A2B5, α-GFAP, or α-GalC. After 1 day in culture all A2B5$^+$ cells were and α-GFAP$^-$/α-GalC$-$.

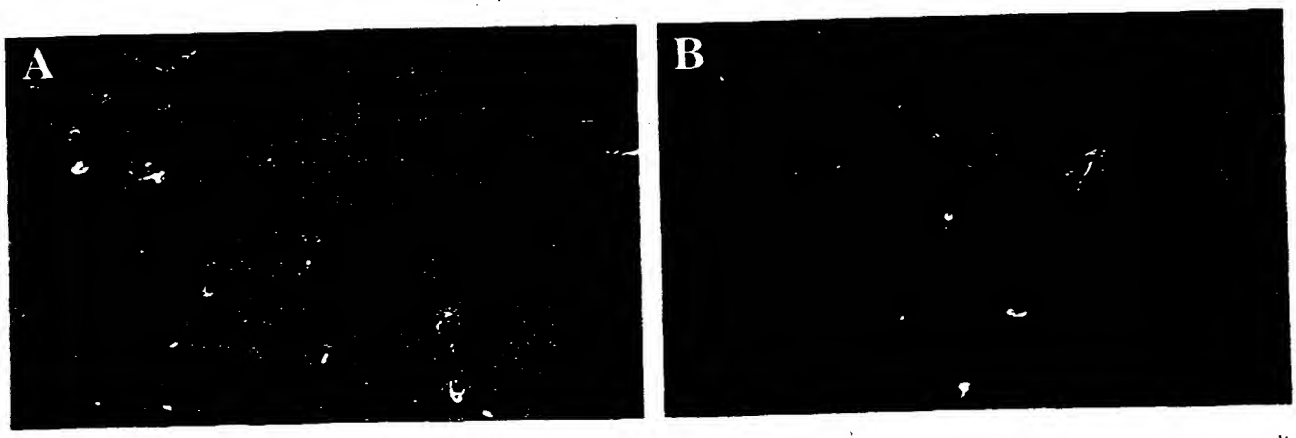

FIG. 7. A2B5$^+$ cells differentiate into oligodendrocytes and astrocytes but not into neurons. A2B5-panned cells were grown on coverslips in the presence of PDGF (A) or bFGF (B) for 9 days. Cultures were then stained with A2B5 (coumarin), α-GalC (rhodamine), α-GFAP (fluorescein), and α-β-III tubulin (rhodamin). Cultures contained oligodendrocytes, astrocytes, and A2B5$^+$ cells but no neurons.

TABLE 3
Individual A2B5⁻ Cells Are Multipotential

Marker expressed	Growth condition: PDGF	Growth condition: FGF CNTF
A2B5⁺/GFAP⁺	0	6
A2B5⁺	51	6
GFAP⁺	51	4
GalC⁺	51	1
β-III tubulin⁺	0	0
Total number of clones	51	6

Note. NEP-derived A2B5⁺ cells were immunopurified and plated at serial dilution in 96-well plates. Individual A2B5 immunoreactive cells were identified and expanded in PDGF/bFGF for 7 days, before replacing the culture medium with DMEM-BS supplemented with PDGF or bFGF/CNTF. After 7 days clones were stained. All clones were labeled with all antibodies in one staining procedure (see Material and Methods).

panded in PDGF/bFGF and then switched to bFGF/CNTF. Surprisingly, all six clones contained cells which were A2B5⁺/GFAP⁺, resembling the type-2 astrocyte phenotype. Only 1 clone contained GalC⁺ oligodendrocytes and no clone contained β-III tubulin⁺ cells (Table 3). This result suggested that in the presence of CNTF and bFGF, A2B5⁻ cells predominantly differentiate into cells with a type-2 astrocytic phenotype.

We also analyzed five A2B5⁺ clones in different neuron promoting conditions and, as before, were unable to generate neurons. Five PDGF/bFGF expanded clones were trypsinized, divided into two portions, and replated into either bFGF alone or bFGF supplemented with retinoic acid. Clones were stained with the antibodies A2B5, α-GFAP, α-GalC, and α-β-III tubulin (Table 4). None of the clones, regardless of whether cells were grown in bFGF alone or bFGF/RA, contained β-III tubulin⁺. In contrast, all five clones consisted of a mixture of cells that were either A2B5⁺ or GFAP⁻, but not both. Only one clone grown in bFGF alone contained GalC⁺ oligodendrocytes, while in bFGF/RA no GalC⁺ oligodendrocytes were found. These data support the initial observation, that A2B5⁺ cells isolated from induced NEP cell cultures were multipotential and restricted in their differentiation potential to cells of the glial lineages.

A2B5⁺ Cells Have an Extended Self-Renewal Potential

In order to fulfill the criteria of a true intermediate precursor, cells need to have an extended self-renewal capacity without losing the ability to differentiate into more than one specific cell type.

To test the self-renewal capacity of individual A2B5⁺ cells, we selected two clones expanded in PDGF/bFGF for 7 days for long-term culture and passaging. The two clones were refed every other day with PDGF/bFGF and maintained for a total of 3 months with 4 serial passages. Clones were grown in PDGF/bFGF as this combination of cytokines apparently inhibited differentiation and promoted division. Cells were stained before and after each passage and were negative for differentiation markers but A2B5⁻ at all time points. To determine the differentiation potential of long term clones, during each passage single cells were replated, reexpanded to 50–200 cells, and switched to PDGF alone to promote differentiation. In this secondary cultures oligodendrocytes and astrocytes appeared consistently after 8–10 days (Fig. 9). The ability to differentiate into oligodendrocytes and astrocytes was not altered significantly with increased passages, suggesting that these long-term propagated cells were still multipotential.

Our results show that A2B5⁺ cells that differentiate from multipotent NEP cells can be expanded and propagated as precursor cells. Passaged individual A2B5⁻ cells self-renew and are able to generate oligodendrocytes, A2B5⁺, and A2B5⁻ astrocytes, but not neurons. NEP-derived A2B5⁺ cells thus represent multipotential intermediate precursor cells restricted to glial lineages.

DISCUSSION

We have shown that multipotent NEP cells can be induced to generate self-renewing precursor cells restricted to subsequent glial differentiation. This self-renewing precursor population can be isolated by immunopanning using the monoclonal antibody A2B5 and can be maintained in an undifferentiated state over multiple divisions when grown in PDGF and bFGF. A2B5⁺ cells differ from the parental NEP cell population in antigenic phenotype and differentiation potential. A2B5⁺ cells lack the ability to differentiate into neurons under conditions that promote neuronal differentiation in NEP cells. A2B5⁺ cells retain, however, the ability to differentiate into oligodendrocytes and astrocytes and are thus identified as multipotential precursors (Fig. 10).

Several lines of evidence show that the A2B5 immunoreactive glial-restricted precursors arise from multipotent NEP cells; (i) NEP cells are a homogeneously nestin⁺/A2B5⁻ population of cells; (ii) Clonal analysis of NEP cell cultures fail to reveal clones that give rise only to glial cells; (iii) A2B5⁺ cells always arise in clones that contain A2B5⁻ neurons and astrocytes. Thus we can find no evidence that the NEP cell population contains a committed A2B5⁻ O2A progenitor. A glial-restricted A2B5⁻ precursor cells ("pre-O2A") has been described by (Grinspan *et al.*, 1990). This "pre-O2A" precursor, however, can be distinguished from NEP cells in its proliferation response to PDGF and its inability to differentiate into neurons. We suggest that this A2B5⁻ cell type could represent an additional intermediate

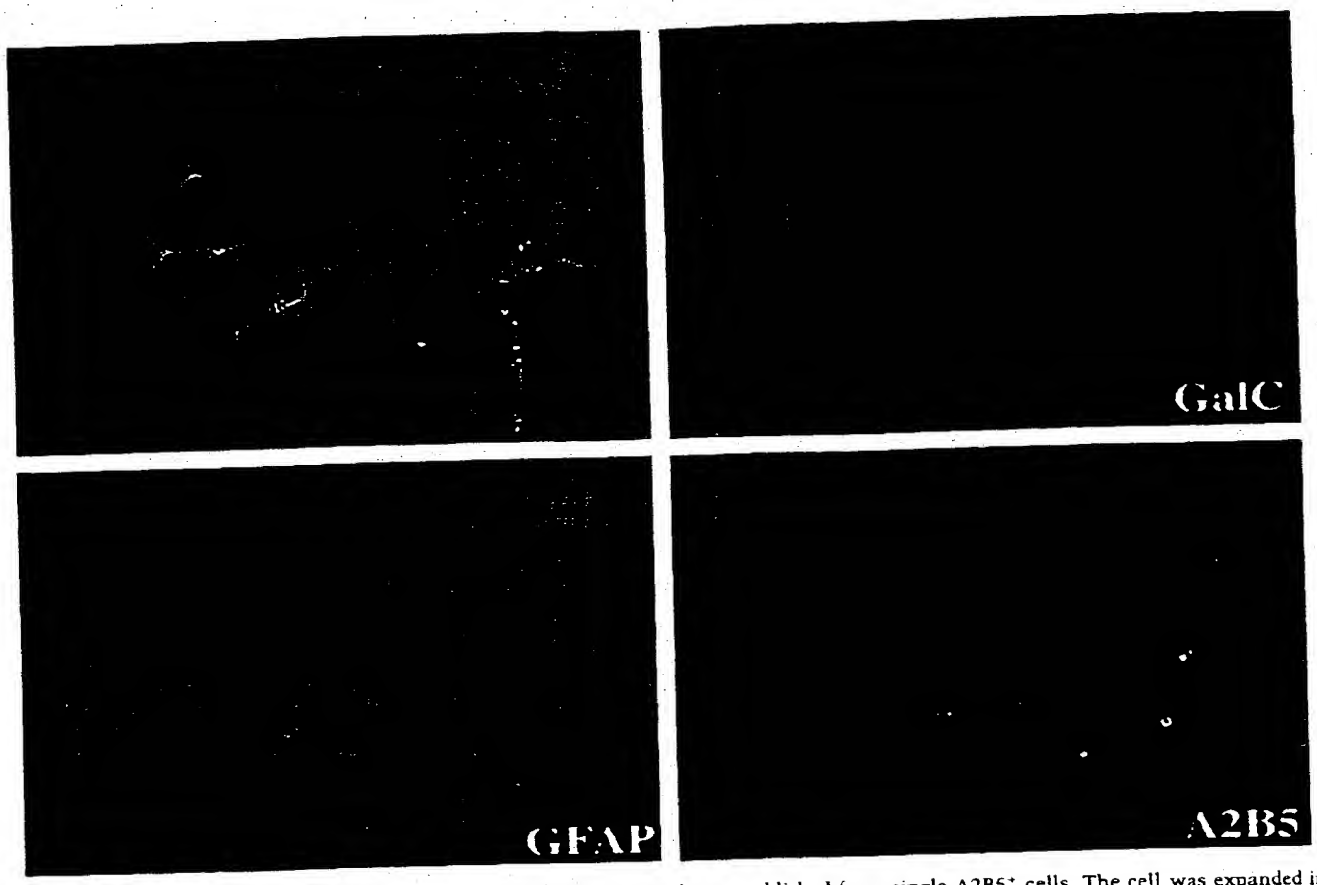

FIG. 8. A2B5$^+$ cells are multipotential. Shown is a representative clone established from single A2B5$^+$ cells. The cell was expanded in PDGF/bFGF to a clonal size of 50–200 cells before the culture was washed with bFGF-free DMEM-BS and further grown in medium supplemented with PDGF. After 8 days clones were stained with A2B5 (rhodamine), α-GalC (coumarin), α-GFAP (fluorescein), and α-β-III tubulin (coumarin). A total of 51 clones was analyzed (Table 3).

step involved in the transition from NEP cells to A2B5$^+$ cells.

The possibility that NEP cells contain subpopulations which are A2B5$^-$ that give rise to precursor populations with other kinds of restrictions cannot be ruled out by our present experiments. Retroviral lineage tracing experiments have suggested the existence of oligodendrocyte-neuron precursor cells in addition to neuron-astrocyte or oligodendrocyte-astrocyte precursor cells (Price *et al.*, 1991; 1992; Williams, 1995; Williams *et al.*, 1991). While we cannot identify putative oligodendrocyte-neuron precursor cells in the A2B5$^+$ population, such cells may well exist in our culture conditions among the A2B5$^-$ population. We did indeed observe the generation of oligodendrocytes and neurons when the A2B5$^-$ supernatant from the immunopanning procedure was cultured. These differentiated cells, however, could also have been the progeny of undifferentiated NEP cells. Without additional markers for either NEP cells or A2B5$^-$ oligodendrocyte-neuron progenitor cells this issue cannot be resolved yet. Alternatively it is possible that the oligodendrocyte-neuron precursor cells previously described are multipotent NEP cells that have the capacity to produce all three cell types (oligodendrocytes, neurons, and astrocytes) but do not differentiate into astrocytes due to environmental restrictions and therefore appear to be restricted to an oligodendrocyte-neuron pathway. In addition a true oligodendrocyte-neuron restricted precursor cell could represent a specialized precursor cell pool restricted to the ventricular zone of the cortex.

An important characteristic of the NEP-derived A2B5$^-$ population is the inability to generate neurons under condition where the parent population generates a large number of neurons. For example, 10 of 10 clones of the parent population grown in the presence of bFGF/RA contain β-III tubulin$^+$ neurons compared to 0 of 5 clones of the A2B5$^-$ population. It is noteworthy that the A2B5$^+$ cells when

TABLE 4
Individual A2B5$^-$ Cells Cannot Generate Neurons

Marker expressed	Growth condition: FGF	FGF RA
A2B5$^-$/GFAP$^-$	0	0
A2B5$^-$	5	5
GFAP$^-$	5	5
GalC$^+$	1	0
β-III tubulin$^+$	0	0
Total number of clones	5	5

Note. NEP-derived A2B5$^+$ cells were immunopurified and plated at limiting dilution in 96-well plates. Individual A2B5 immunoreactive cells were identified and expanded in PDGF/bFGF. After 7 days clones were switched to bFGF or bFGF/RA and stained 7 days later. All clones were labeled with the indicated antibodies in one staining procedure (see Materials and Methods).

grown in the presence of retinoic acid (RA) did not only fail to generate neurons, but did also not differentiate into oligodendrocytes. This result is consistent with observations published by Noll and Miller (1994). Although the A2B5$^+$ cell population appears morphologically homogenous and uniform in its antigenic phenotype, it nevertheless remains to be determined whether this population is also truly homogenous in its differentiation potential. All A2B5$^+$ cells that could be clonally expanded in the presence of PDGF and bFGF were multipotential and gave rise to both astrocytes and oligodendrocytes. Whether unipotent cells exist that require other expansion conditions remains a possibility. We note, however, that less than 10% of the A2B5 population underwent cell death when cells were expanded in PDGF and bFGF. Thus, if additional glial restricted precursors exist they likely represent a small fraction of the A2B5 population.

Our clonal analysis of A2B5 immunoreactive cells shows that single A2B5$^+$ cells can be expanded and propagated in a mixture of PDGF/bFGF without losing their differentiation potential. Expanded clones can differentiate into astrocytes and oligodendrocytes but not into neurons. The culture condition determines what glial differentiation pathways are chosen by a clone. PDGF promotes the differentiation into oligodendrocytes, whereas bFGF promotes astrocytic differentiation. The presence of CNTF leads to the coexpression of A2B5 and GFAP in the majority of cells. This appearance of the type-2 astrocytic phenotype could resemble a preference for a specific astrocytic differentiation pathway promoted by CNTF. Whether A2B5$^+$ astrocytes generated in the presence of CNTF represent a functionally distinct phenotype from the A2B5$^-$ astrocytes generated in the presence of bFGF remains to be determined.

Comparison of the NEP-derived A2B5$^+$ precursor with other glial-restricted precursors identified in the CNS (Aloisi *et al.*, 1992; Blau and Hughes, 1990; Cameron and Rakic, 1991; Chan *et al.*, 1990; Cochard and Giess, 1995; Davis and Temple, 1994; Elder *et al.*, 1988; Fok-Seang and Miller, 1994; Fulton *et al.*, 1992; Galileo *et al.*, 1990; Gard *et al.*, 1995; Grinspan *et al.*, 1990; Hardy and Reynolds, 1991; Hardy and V. L. Friedrich, 1996; Knapp, 1991; Luskin *et al.*, 1993; Miller, 1996; Ono *et al.*, 1995; Raff *et al.*, 1983; Rivkin *et al.*, 1995; Wood and Williams, 1984) reveals several similarities and differences. NEP-derived A2B5$^+$ cells share several characteristics with optic nerve-derived O-2A progenitor cells, including morphology, migratory nature, responsiveness to PDGF and bFGF, and the ability to generate oligodendrocytes and type-2 astrocytes. In contrast to postnatal O-2A progenitor cells, however, NEP-derived A2B5$^+$ cells can also give rise to astrocytes that are GFAP$^-$/A2B5$^-$ (and therefore not Type 2 astrocytes). Thus, it is possible, that A2B5$^+$ cells purified from NEP cells represent an earlier stage of glial precursor cell development than the A2B5$^+$ O-2A progenitor cells that have been studied so extensively.

Previous studies have also identified the existence of A2B5$^+$ precursor populations in the spinal cord which are able to generate oligodendrocytes and astrocytes (Fok-Seang

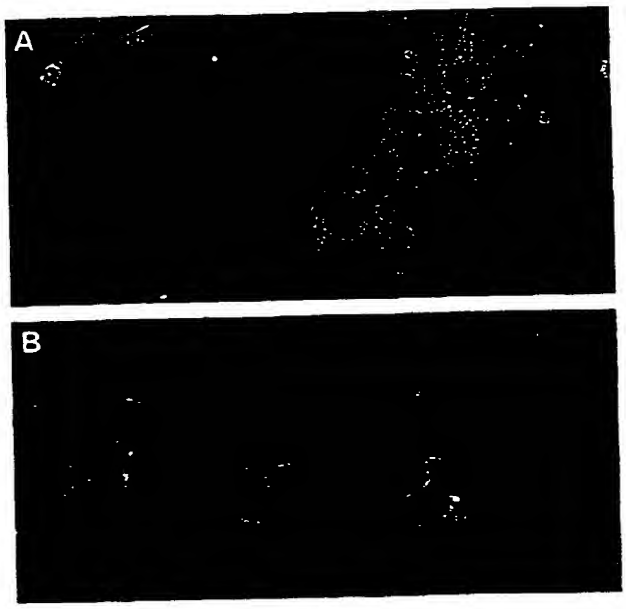

FIG. 9. A2B5$^+$ cells can be propagated without losing their differentiation potential. (A) Phase contrast picture of a long-term clone grown in PDGF/bFGF for 3 months after four passages. (B) An aliquot of this clone was replated after trypsinization on a coverslip, grown in medium supplemented with PDGF, and stained after 10 days with A2B5 (coumarin), α-GFAP (fluorescein), α-GalC (rhodamine), and α-β-III tubulin (rhodamine). The clone contained a mixed population of glial cells but no neurons.

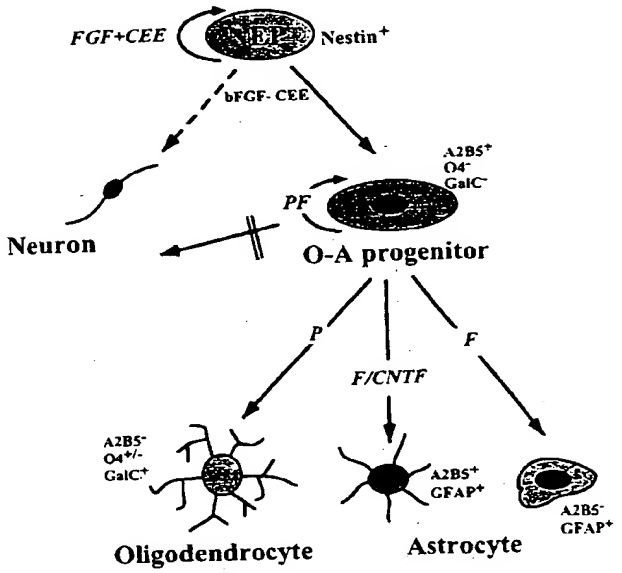

FIG. 10. Model for the generation of glial cells from multipotent NEP cells. While neuroepithelial cells (NEP), which can be expanded in the presence of FGF and chick extract (CEE) are not restricted in their differentiation potential to specific lineages upon CEE withdrawal, A2B5$^+$ cells which arise from this NEP cell population progress to a stage where they lose the potential to generate neurons, but are still able to give rise to oligodendrocytes (O), a pathway promoted by platelet-derived growth factor (P), and antigenically different types of astrocytes (A) depending on the presence of CNTF. These O-A progenitor cells can be expanded in the presence of a combination of P and F.

and Miller, 1992, 1994; Warf *et al.*, 1991). Our results confirm and extend these observations by demonstrating the transition from undifferentiated NEP to at least bipotential A2B5$^+$ cells. It seems likely that at least a subpopulation of NEP-derived A2B5$^-$ cells described here is identical to previously described A2B5$^+$ cells present in embryonic spinal cord.

In summary, we have provided direct evidence for a lineage relationship between multipotent and lineage-restricted precursor cell populations and have identified morphological, antigenic, and cytokine dependence data to distinguish between the two populations. The data presented show for the first time that the transition of multipotent cells to terminally differentiated cells involves the generation of more restricted precursor cells. Equally importantly we have established an accessible culture system to follow the development of isolated precursor cells and to study the cellular and molecular events that regulate differentiation processes. It remains to be determined whether this paradigm is used by early stem cell populations derived from regions other than the spinal cord.

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The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification

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Summary

OLIG1 and OLIG2 are basic-helix-loop-helix (bHLH) transcription factors expressed in the pMN domain of the spinal cord, which sequentially generates motoneurons and oligodendrocytes. In *Olig1/2* double-mutant mice, motoneurons are largely eliminated, and oligodendrocyte differentiation is abolished. Lineage tracing data suggest that *Olig1*$^{-/-}$*2*$^{-/-}$ pMN progenitors instead generate V2 interneurons and then astrocytes. This apparent conversion likely reflects independent roles for OLIG1/2 in specifying motoneuron and oligodendrocyte fates. *Olig* genes therefore couple neuronal and glial subtype specification, unlike proneural bHLH factors that control the neuron versus glia decision. Our results suggest that in the spinal cord, *Olig* and proneural genes comprise a combinatorial code for the specification of neurons, astrocytes, and oligodendrocytes, the three fundamental cell types of the central nervous system.

Introduction

The three fundamental cell types of the vertebrate central nervous system (CNS) are neurons, astrocytes, and oligodendrocytes. This basic triad comprises many hundreds or even thousands of distinct neuronal subtypes, in addition to subtypes of astroglia and perhaps of oligodendroglia as well (Raff, 1989; Woodruff et al., 2001). The molecular mechanisms by which these diverse neural cell types are properly generated in space and time are incompletely understood. In recent years, a great deal has been learned about the transcriptional control of the neuron-glial fate decision (Tomita et al., 2000; Nieto et al., 2001; reviewed in Vetter, 2001) and about the control of neuron subtype specification (Briscoe et al., 2000; Jessell, 2000). Rather less is known, however, about the transcriptional control of glial subtype determination.

Two major classes of transcription factors have emerged as determinants of neuron versus glial fate determination and of neuron subtype specification: the basic-helix-loop-helix (bHLH) factors (Vetter, 2001) and homeodomain (HD) factors (Jessell, 2000), respectively. In vertebrates, bHLH factors homologous to the *Drosophila* proneural genes, such as the *Neurogenins (Ngns)* (Gradwohl et al., 1996; Ma et al., 1996; McCormick et al., 1996) and *Mash1* (Johnson et al., 1990), promote neuronal differentiation at the expense of the glial fate (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b). In the spinal cord, a combinatorial code of HD transcription factors specifies the regional identity of progenitor domains along the dorso-ventral axis (Briscoe et al., 2000; Jessell, 2000). Motoneurons are generated from the pMN domain, while V0, V1, V2, and V3 interneurons are generated from the p0, p1, p2, and p3 domains, respectively (Briscoe et al., 2000; Jessell, 2000). This discontinuous patterning arises from mutually repressive interactions between the HD factors that specify adjacent progenitor domains (Briscoe et al., 2000; Muhr et al., 2001).

Recently, we and others identified a subclass of neural bHLH factors, called *Olig* genes (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). In the mouse, there are two *Olig* genes that are specifically expressed in oligodendrocyte precursors, called *Olig1* and *2* (Lu et al., 2000; Zhou et al., 2000), while in the chick a single gene orthologous to *Olig2* has been identified (Mizuguchi et al., 2001; Zhou et al., 2001). In the spinal cord, oligodendrocyte precursors emerge from a highly restricted domain of the ventral ventricular zone (Miller, 1996; Richardson et al., 2000). This region is precisely demarcated by expression of *Olig1* and *Olig2* (Lu et al., 2000; Zhou et al., 2000). *Olig2* is sufficient to cause ectopic differentiation of oligodendrocytes in the chick spinal cord when misexpressed together with the HD factor Nkx2.2 (Sun et al., 2001a; Zhou et al., 2001), while *Olig1* promotes oligodendrocyte differentiation in rodent cortex (Lu et al., 2001).

Prior to oligodendrogliogenesis, the domain of *Olig2* expression corresponds to the pMN domain, from which motoneurons are generated (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001). Gain-of-function experiments suggest that OLIG2 plays a determinative role in patterning the pMN domain and also initiates motoneuron differentiation and cell cycle arrest, in part by promoting expression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). These data suggest that OLIG2 sequentially controls both motoneuron and oligodendrocyte fate determination. Interestingly, the bHLH factor appears to function in both cases as a transcriptional repressor (Novitch et al., 2001; Zhou et al., 2001).

To rigorously assess the requirement for *Olig* genes in motoneuron and oligodendrocyte differentiation, we have generated double-homozygous mice lacking both *Olig1* and *Olig2*. In *Olig1/2* double mutants, presumptive motoneuron precursors are transformed into V2 interneuron precursors, and oligodendrocytes are lost throughout the brain and spinal cord. Surprisingly, many *Olig2*-expressing oligodendrocyte precursors are transformed into astrocytes. Thus, in the absence of *Olig1/2* function, the sequential production of motoneurons and oligodendrocytes is converted into the sequential production of interneurons and astrocytes. These data suggest that *Olig* genes couple neuronal and glial subtype specification.

Results

Generation of *Olig1* and *Olig2* Double-Mutant Mice

The coexpression of *Olig1* and *Olig2* in vivo (Zhou et al., 2000) raised the possibility that deletion of either of

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these genes alone might be compensated by the function of the other. In order to circumvent this problem, we decided to generate an *Olig1* and *Olig2* double mutant. The mouse *Olig1* and *Olig2* genes are tightly linked on chromosome 16, about 36 kb from each other (data not shown). In order to preserve the *Olig1-Olig2* intergenic region, double-mutant mice were generated through two rounds of homologous recombination in ES cells. The *Olig2* coding region was replaced by a targeting cassette composed of a histone-GFP (hGFP) fusion (Kanda et al., 1998) and PGK-neomycin (Figures 1A and 1B), while the *Olig1* coding region was replaced by a tau-LacZ and PGK-hygromycin cassette (Figures 1D and 1E).

Using a Cre/lox-mediated analytic strategy (see Experimental Procedures), two ES clones were identified in which the *Olig1* and *Olig2* targeted loci lay in *cis* (Figure 1H). Sister cells from these clones, unmodified by Cre recombinase, were injected into blastocysts, and the targeted alleles were transmitted through the germ line (Figures 1C and 1F). Double-heterozygous mice were born at the expected Mendelian frequency and were viable and fertile. However, no live births of homozygous mice were observed, and starting from E18.5, the homozygous embryos appeared smaller than their littermates.

We initially examined the pattern of GFP and LacZ expression in heterozygous $Olig1^{+/-}Olig2^{+/-}$ embryos between E9.5 and E16.5. At E9.5, GFP was strongly expressed in a ventral domain of the spinal cord that corresponds to pMN (Figure 1I), similar to the pattern of endogenous *Olig2* expression (Figure 1J; also see Takebayashi et al., 2000). In contrast, only a few cells were observed to be weakly lacZ positive at this stage (Figure 1N), in agreement with the relatively weak expression of endogenous *Olig1* during the period of neurogenesis (Figure 1O). At E16.0, when *Olig2* expression is restricted to oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000), nearly all OLIG2-positive cells were also GFP-positive (Figures 1K–1M, arrows). In addition, GFP colocalized extensively with the *Olig1* knockin marker tau-lacZ (Figures 1P–1R, arrows), consistent with the coexpression of endogenous *Olig1* and *2* in oligodendrocyte precursors (Zhou et al., 2000). Thus, in heterozygous $Olig1^{+/-}Olig2^{+/-}$ mice, the expression of GFP and tau-lacZ faithfully recapitulates the pattern of endogenous *Olig2* and *Olig1* expression in cells of both the motoneuron and oligodendrocyte lineages.

Deletion of *Olig2* and *Olig1* Results in Loss of Motoneurons and a Concomitant Ventral Expansion of V2 Interneurons

We first focused our analysis of *Olig1/2* double-mutant embryos on the generation of several neuronal subtypes derived from the four ventral-most progenitor domains of the spinal cord: $En1^+$ V1 interneurons, $Chx10^+$ V2 interneurons, $Isl1/2^+$ and $Hb9^+$ motoneurons, and $Ngn3^+$ V3 interneurons (Figures 2A–2E). Most $Isl1/2^+$, $Hb9^+$ motoneurons were lost at all axial levels of the homozygous mutant spinal cord at E10.5 (Figures 2H, 2I, and 2K, white bars), while the number of such motoneurons was the same in heterozygote and wild-type (Figures 2C, 2D, and 2K, dark and light gray bars). The loss of motoneurons in the $Olig1^{-/-}Olig2^{-/-}$ spinal cord was not due to cell death, as no increased apoptosis was detected by the TUNEL assay at this stage (Figures 3O and 3T). Although very few presumptive motoneurons ($Isl1/2^+$, $Hb9^+$ cells) were detectable at E10.5 in $Olig1^{-/-}Olig2^{-/-}$ embryos (Figures 2H and 2I, arrowheads), it was possible that a normal number of these neurons might be recovered at later stages through compensatory mechanisms. However, at E13.5, neither somatic (Figures 2L and 2M, arrows) nor visceral (Figures 2L and 2M, arrowheads) motoneurons were detected in the $Olig1^{-/-}Olig2^{-/-}$ spinal cord (Figures 2P and 2Q, arrows and arrowheads, and 2T, white bars). Moreover, no projecting axons were observed in the ventral root (Figures 2O and 2S, arrows), consistent with a lack of both classes of spinal motoneurons.

In contrast to the dramatic loss of motoneurons, the number of $Chx10^+$ cells, which derive from the p2 domain just dorsal to the pMN domain, was increased by about 80% in the double-null mutant spinal cord at E10.5 (Figures 2B versus 2G; 2K, Chx10, white bar). Furthermore, many $Chx10^+$ cells occupied a more ventral position, in territory normally occupied by motoneurons (Figure 2G, yellow arrowhead). The increased number and ventral expansion of $Chx10^+$ V2 interneurons were also apparent at E13.5 (Figures 2N, 2R, and 2T). The number and distribution of $En1^+$ V1 interneurons and $Ngn3^+$ V3 interneurons, by contrast, were largely unaltered in the mutant (Figures 2A, 2E, 2F, 2J, and 2K).

The preceding data suggested that in the absence of *Olig1/2* function, pMN progenitors might give rise to V2 interneurons instead of motoneurons. To confirm this, we used the *Olig2* knockin marker hGFP as a short-term lineage tracer to compare the identities of the neuronal progeny derived from the pMN domain of heterozygous versus homozygous *Olig1/2* double-mutant embryos. In heterozygotes, *Olig2-hGFP*-derived precursors gave rise to $Islet1/2^+$ motoneurons (Figure 3A, yellow cells) but not $Chx10^+$ V2 interneurons (Figure 3B). By contrast, in the homozygotes there were many GFP^+, $Chx10^+$ cells present in the marginal zone lateral to the pMN domain of the ventricular zone (Figure 3G, yellow cells). At no time did we detect any $Islet1/2^+Chx10^+$ phenotypically hybrid cells (data not shown). In the immediately overlying p2 domain, $Chx10^+GFP^-$ V2 interneurons were produced in both heterozygotes and homozygotes (Figures 3B and 3G, white arrowheads). These data strongly suggest that precursor cells from the pMN domain of *Olig1/2* homozygous animals generate V2 interneurons instead of motoneurons.

Irx3 Is Derepressed in pMN in the Absence of *Olig2* and *Olig1* and Respecifies pMN to p2

The loss of motoneurons and concomitant ventral expansion of V2 interneurons in the double-null mutant could reflect a conversion of the pMN domain to a p2 identity. Consistent with this idea, the expression of Irx3, a p2 domain patterning molecule (Briscoe et al., 2000), expanded into the pMN domain in $Olig1^{-/-}Olig2^{-/-}$ double mutants at E10.5 (Figures 3C and 3H, arrows). Pax6 expression, which is high in the p2 domain but lower in the pMN domain (Figures 3D, arrow, and 3E), also

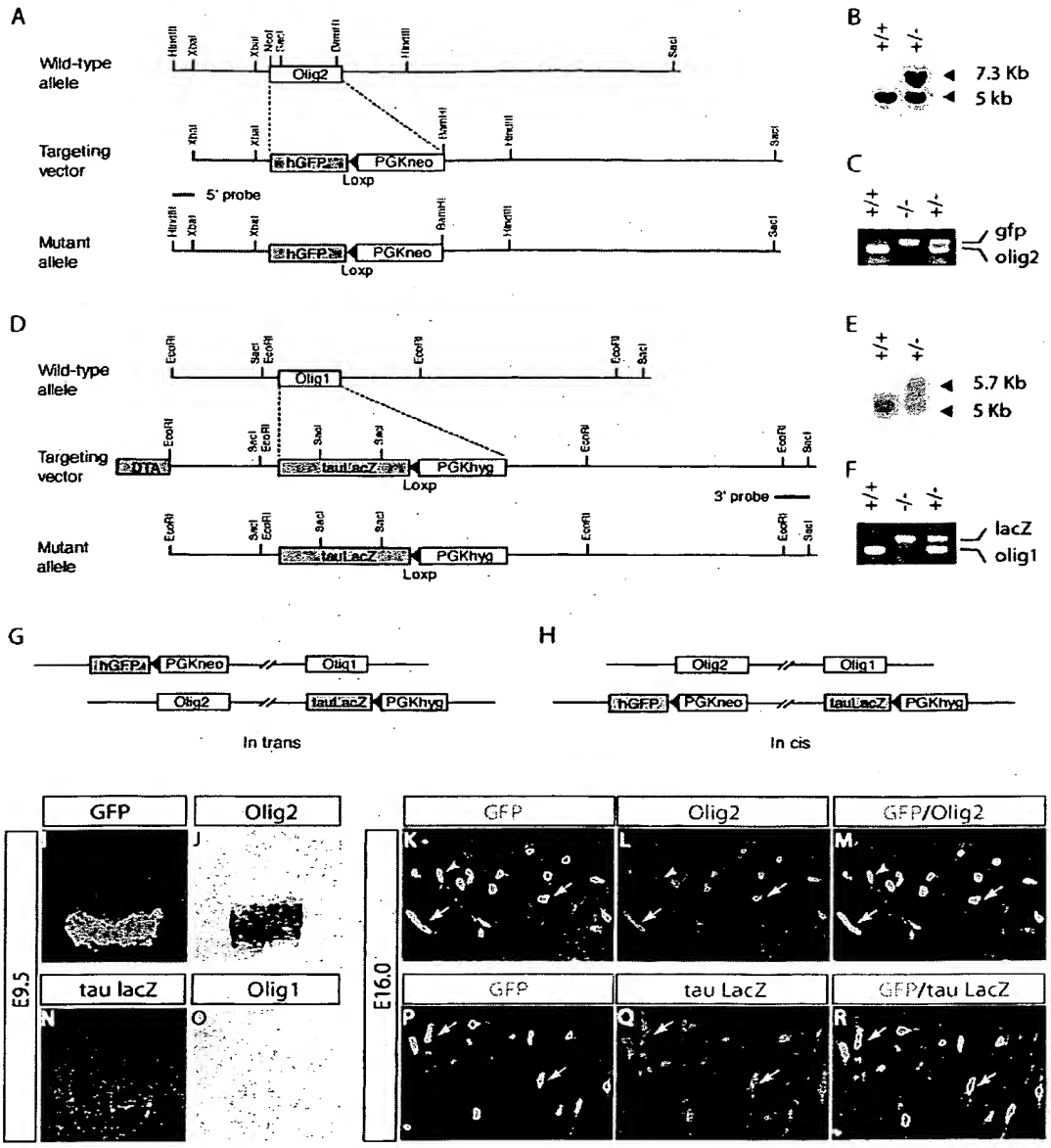

Figure 1. Inactivation of *Olig1* and *Olig2* by Homologous Recombination

(A–C) First round of homologous recombination at the *Olig2* locus.

(A) A *Histone-EGFP/loxp/PGK* neomycin cassette replaced the *Olig2* coding region.

(B) Correct recombination at *Olig2* locus verified by Southern blot analysis of ES clones.

(D–F) Second round of homologous recombination at the *Olig1* locus.

(D) A *tau-LacZ/loxp/PGK* hygromycin cassette replaced the *Olig1* coding region in ES cells in which one *Olig2* locus had been successfully targeted. Abbreviation: DTA, diphtheria toxin A chain.

(E) Correct recombination at the *Olig1* locus verified by Southern blot.

(C and F) Successful germline transmission of the targeted *Olig2* and *Olig1* alleles in *Olig1,2* double-mutant embryos confirmed by genotyping with specific PCR primers.

(G and H) Schematic diagram showing that the two targeting cassettes could lie either in *trans* (G) or in *cis* (H) to each other. A Cre/Loxp analysis was used to identify ES cells in which the two cassettes lie in *cis* (see Experimental Procedures).

(I, J, N, and O) Expression of histone-EGFP and tau-LacZ in the heterozygotes. Thoracic spinal cord sections of E9.5 *Olig1*$^{+/-}$*Olig2*$^{+/-}$ heterozygous embryos were either labeled with anti-GFP antibody (I) or anti-LacZ antibody (N) or were probed by in situ hybridization with cRNAs against Olig2 (J) and Olig1 (O).

(K–M and P–R) Thoracic spinal cord sections from E16.0 *Olig1*$^{+/-}$*Olig2*$^{+/-}$ heterozygous embryos were double labeled with antibodies to GFP and Olig2 (K–M) or to GFP and lacZ (P–R). Extensive colocalization of GFP and Olig2 (K–M) as well as GFP and tau LacZ (P–R) were observed. Arrows indicate double-positive cells. A small number of GFP^{+} cells was found to be Olig2^{-} at this stage (K–M, arrowheads).

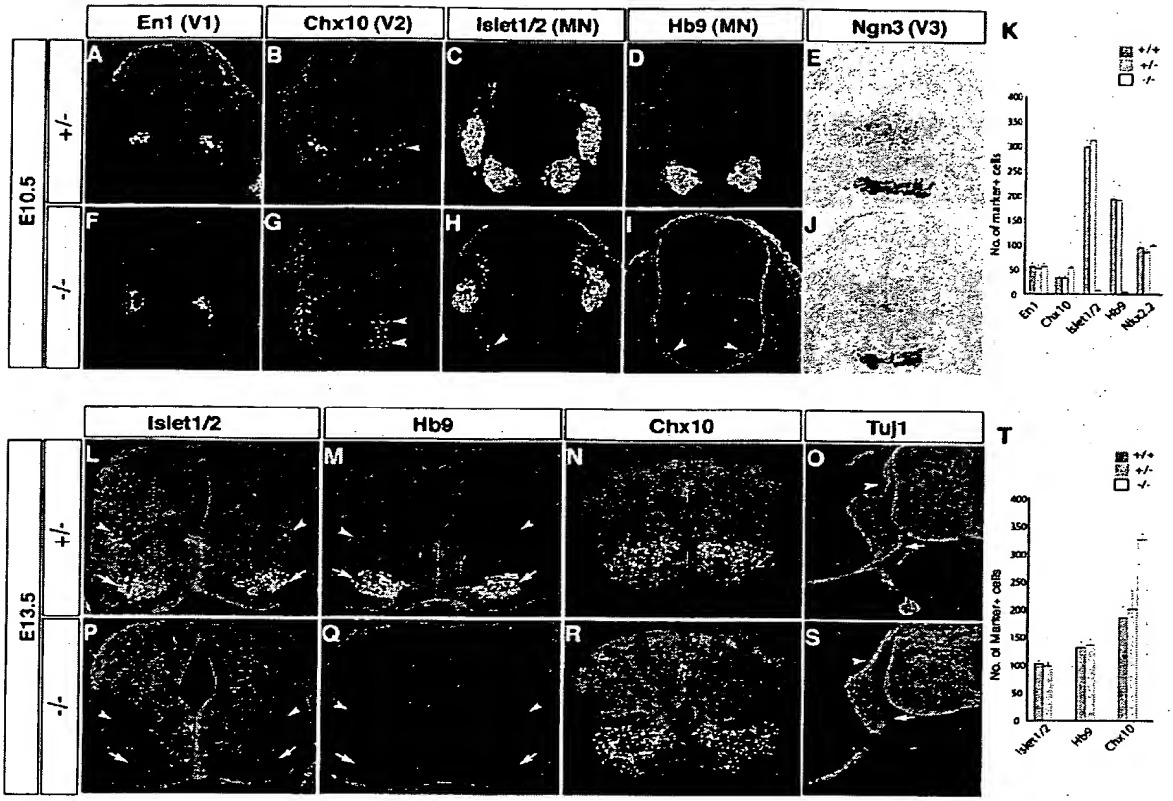

Figure 2. Loss of Isl1/2$^+$Hb9$^+$ Motoneurons and Concomitant Ventral Expansion of Chx10$^+$ V2 Interneurons in the Absence of *Olig1* and *Olig2*

(A–K) Crosssections of E10.5 thoracic spinal cord were stained with antibodies to detect four types of ventral neurons: En1$^+$ V1 interneurons (A and F), Chx10$^+$ V2 interneurons (B and G, arrowheads), Isl1/2$^+$ and Hb9$^+$ motoneurons (C, D, H, and I), and Ngn3$^+$ V3 interneurons (E and J). Note the dramatic reduction of Isl1/2$^+$ and Hb9$^+$ motoneurons in the homozygous spinal cord (C, D, H, and I), while the Chx10$^+$ V2 neurons increased in number and expanded ventrally (B and G, arrowheads). Arrowheads in (H) and (I) indicate the few residual motoneurons formed in the homozygote. White and yellow arrowheads in (B) and (G) indicate Chx10$^+$ V2 interneurons in the p2 and pMN domains, respectively. Quantitative analysis is shown in (K). The number of marker positive cells is presented as mean ± S.D. from nine sections of three embryos. Isl1/2$^+$Hb9$^+$ motoneurons decreased to <5%, and Chx10$^+$ V2 neurons increased ~80% in the double-null mutant compared to heterozygote or wild-type.

(L–T) Motoneuron phenotype at E13.5. Both visceral (L and M, arrowheads) and somatic (L and M, arrows) motoneurons were lost in the homozygotes (P and Q, arrowheads and arrows). In addition, projecting axons were selectively lost in the ventral root of the null mutant (O and S, arrows). Quantitative analysis revealed a 60% increase in the number of Chx10$^+$ cells in the double-null mutant (T; mean ± S.D., six sections from 2–3 animals).

increased in the pMN domain of the null mutant, so that cells in both pMN and p2 were now expressing equally high levels of Pax6 (Figure 3I, arrow). The observed ventral expansion of Irx3 is predicted by the observation that Irx3 and OLIG2 exert crossrepressive activities in gain-of-function assays (Novitch et al., 2001). Surprisingly, however, it did not cause a complete loss of GFP expression from the *Olig2* locus (Figures 3P and 3R), perhaps because the repressive effect of Irx3 is overridden by the higher levels of Shh signaling more ventrally. The expression of several other ventral spinal cord patterning molecules, including Dbx2, Nkx6.1, Nkx6.2, and Nkx2.2 (Briscoe et al., 2000), was unchanged in *Olig1/2* double mutants (data not shown). Taken together, these data suggest that in the absence of *Olig1/2*, pMN cells are converted to a p2 identity (Figures 3E and 3J).

Olig2 and *Olig1* Regulate Neurogenin 2 Expression in pMN

Ectopic expression studies in chick suggested that deletion of *Olig2* and *Olig1* should cause a loss of *Ngn2* expression in the pMN domain (Novitch et al., 2001). Consistent with this prediction, no NGN2-positive cells were detected in the presumptive pMN domain of the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ mutant at E10.0 (Figure 3P), while prominent NGN2 expression was evident in the GFP$^+$ pMN domain of the heterozygous spinal cord (Figure 3K, yellow cells). The lack of apoptotic cells detected by TUNEL labeling in the mutant spinal cord (Figures 3O and 3T) suggests that the loss of NGN2$^+$ cells in the pMN domain does not reflect cell death. We also observed a slight ventral expansion of MASH1 into pMN in *Olig1/2* double mutants (Figures 3L versus 3Q, arrow).

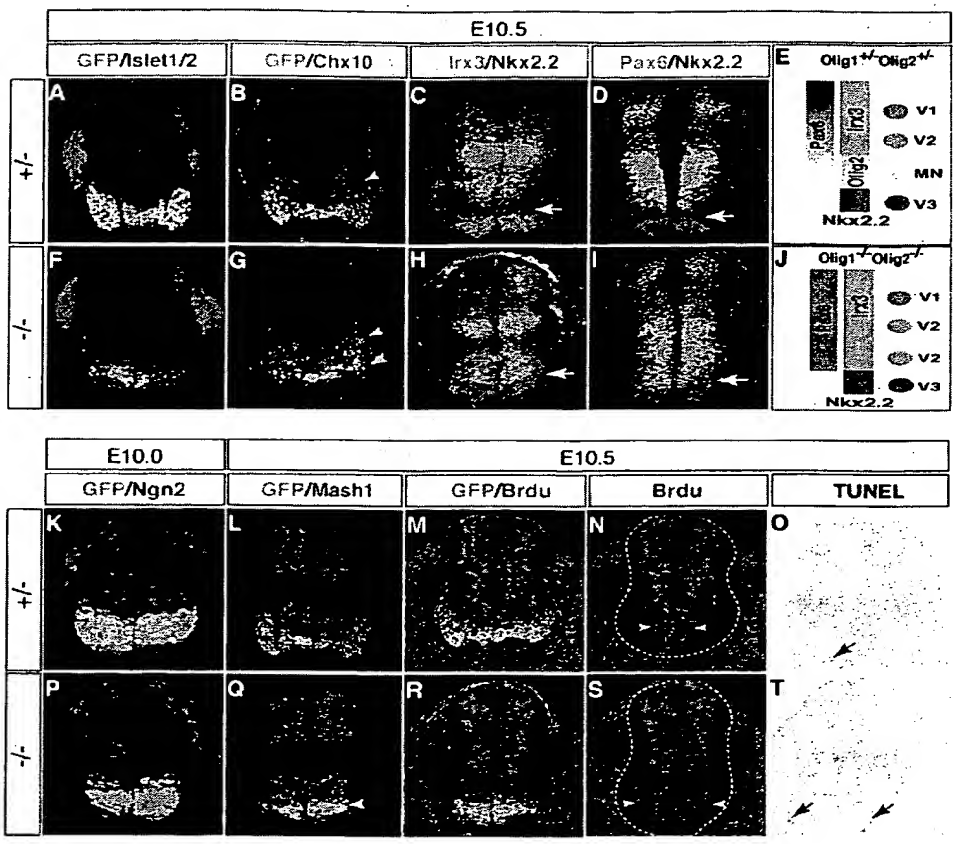

Figure 3. Derepression of *Irx3* and Repression of *Neurogenin2* in the pMN Domain of *Olig1*$^{-/-}$*Olig2*$^{-/-}$ Embryos

(A, B, F, and G) OLIG2-hGFP$^+$ precursors in pMN generate Islet1/2$^+$ motoneurons in heterozygotes (A, yellow cells) but instead produce Chx10$^+$ V2 interneurons in homozygotes (G, yellow arrowhead). White arrowheads in (B) and (G) indicate Chx10$^+$, OLIG2-hGFP$^-$ V2 interneurons generated from the p2 domain.

(C and H) Irx3 is derepressed in the pMN of the mutant spinal cord (H, arrow), as is Pax6 (D and I; arrows indicate pMN domain).

(E and J) Summary of the ventral spinal cord patterning defects in *Olig1/2* mutants.

(K, P) Neurogenin 2 (Ngn2) expression is selectively lost in pMN of the mutant at E10.0, while a delayed expansion of MASH1 into this domain is detected at E10.5 (L and Q, arrowhead).

(M, N, R, and S) Many BrdU$^+$ cells persist outside the ventricular zone of the pMN in the null mutant (S, arrowheads) compared to heterozygotes (N); the same fields with GFP expression superimposed are shown in (R) and (M), respectively.

(O and T) No significant cell death was observed in the spinal cord at this stage (E10.5). Arrows indicate apoptotic cells outside the spinal cord.

As *Mash1* has recently been shown to be necessary and sufficient for Chx10 expression (Parras et al., 2002), these results may explain how V2 interneurons can differentiate from the mutant pMN despite the absence of NGN2 (Scardigli et al., 2001).

Since NGN2 has been shown to promote cell cycle exit and terminal differentiation (Farah et al., 2000; Novitch et al., 2001), we reasoned that the loss of *Ngn2* expression in pMN might cause delayed cell cycle exit by pMN-derived precursors as they migrated from the ventricular zone. To assess this, we measured BrdU incorporation after a 2 hr pulse in vivo at E10.5. An increased number of BrdU$^+$ cells was detected outside the ventricular zone in the GFP$^+$ region of the double-null mutant (Figures 3M, 3N, 3R, and 3S, arrowheads). These data suggest that pMN cells lacking OLIG2 fail to exit the cell cycle before migrating into the marginal zone. The presence of ectopic MASH1$^+$ cells in pMN is not inconsistent with this observation, as we have recently found that MASH1 promotes cell cycle arrest less efficiently than does NGN2 (Lo et al., 2002).

Failure of Oligodendrocyte Development in *Olig1/2* Double Mutants

We next examined the phenotypic consequences of *Olig2* and *Olig1* deletion on the development of oligodendrocytes. To detect oligodendrocyte precursors, *PDGFRα* and *Sox10* were used as markers (Hall et al., 1996; Zhou et al., 2000), while *MBP* and *PLP/DM20* were used to detect mature oligodendrocytes (Zhou et al., 2001). At no time did we detect expression of any of these markers in the *Olig1/2* double-homozygous mutant at all axial levels of spinal cord examined (Figure 4, −/−, and data not shown) as well as in all brain

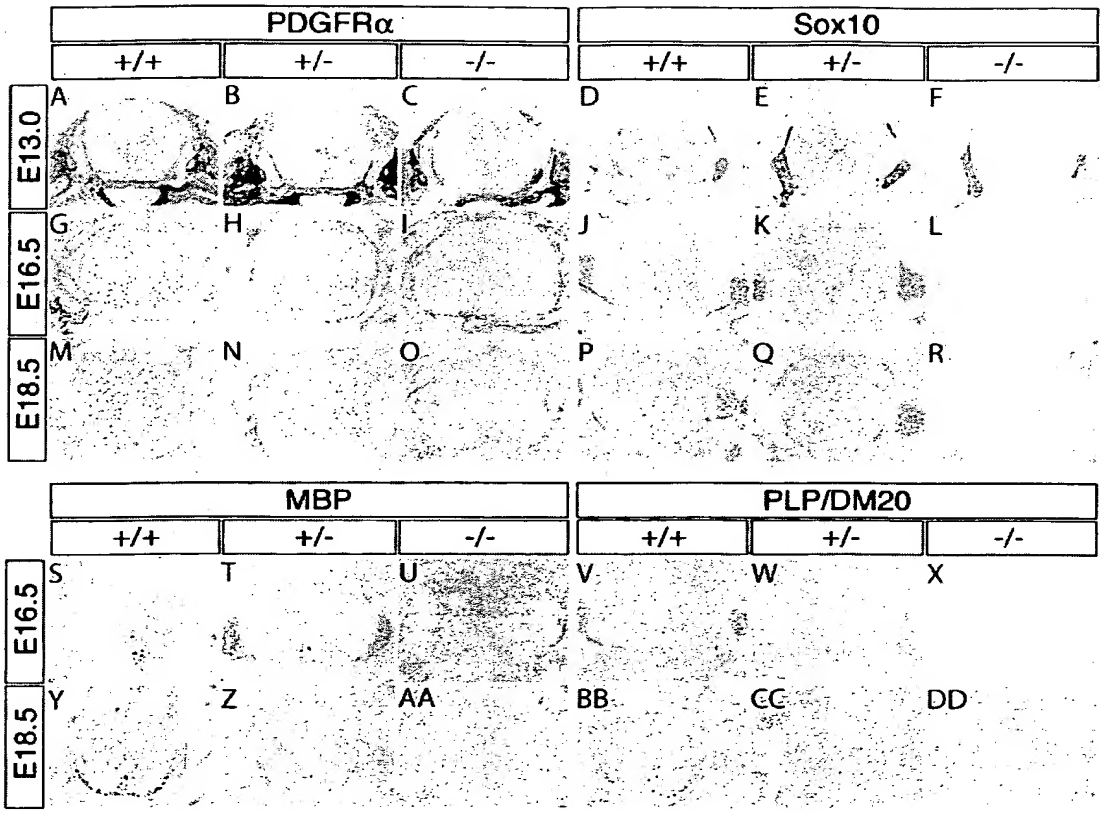

Figure 4. Spinal Cord Oligodendrocytes Fail to Develop in the Absence of *Olig1* and *Olig2*

(A–R) In situ hybridization with the oligodendrocyte precursor markers *PDGFRα* and *Sox10* on crosssections of thoracic spinal cord at indicated stages. Note the total absence of *PDGFRα* and *Sox10* expression in the null mutant spinal cord (C, F, I, L, O, and R).

(S–DD) No *MBP*$^+$ or *PLP/DM20*$^+$ mature oligodendrocytes were detected in null mutant spinal cord (U, X, AA, and DD). In addition, the number of *MBP*$^+$ and *PLP/DM20*$^+$ oligodendrocytes was smaller in the heterozygotes (Z and CC) than in wild-type (Y and BB) at E18.5.

areas examined (Figures 5D–5F and data not shown). In contrast, numerous cells expressing these oligodendrocyte precursor and differentiation markers were present in the wild-type (Figure 4, +/+) and heterozygous (Figure 4, +/−) spinal cord and brain (Figures 5A–5C and data not shown). Thus, there is a total failure of oligodendrocyte formation in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ double mutant.

Cell counts at E16.5 revealed no decrease in the number of *PDGFRα*$^+$ precursors at thoracic levels of the spinal cord in heterozygotes compared to wild-type (297 ± 17 versus 290 ± 19 cells per 18 μm section, respectively; mean ± S.D., n = 6 sections from three embryos). Consistent with these data, in heterozygotes at E16.5 and P8, *MBP* and *PLP/DM20* expression was normal (Figures 4S, 4T, 4V, and 4W and data not shown). Surprisingly, however, between E18.5 and P0, there was significantly less expression of these mature oligodendrocyte markers in the heterozygotes compared to wild-type (Figures 4Y, 4Z, 4BB, and 4CC and data not shown). These data suggest that a full dosage of *Olig* genes is required for the progression of oligodendrocyte differentiation but not for the initiation of this process.

Olig1 Is Functionally Redundant with *Olig2* in Hindbrain Oligodendrocyte Development

We next examined the phenotype of *Olig1/2* double-knockout embryos in the hindbrain. As in the spinal cord, hindbrain somatic motoneuron differentiation did not occur in *Olig1/2* double mutants, as evidenced by the loss of Isl1/2$^+$, Hb9$^+$ cells and of the XIIth cranial somatic motor nerve (Figures 5G–5I versus 5J–5L, arrows). In contrast, visceral motoneurons, identified by coexpression of Isl1/2 and Phox2b (Dubreuil et al., 2000), were generated (Figures 5J–5L, arrowheads). These results are consistent with the fact that visceral motoneurons in the hindbrain derive from the p3 domain (Briscoe et al., 1999), which does not express either *Olig1* or *Olig2* (data not shown).

In *Olig2*$^{-/-}$ single mutants, oligodendrocytes are spared in the hindbrain while they are lost throughout the spinal cord (Lu et al., 2002 [this issue of *Cell*]). In contrast, we found that neither oligodendrocyte precursors nor mature oligodendrocytes were generated in the hindbrain of *Olig1/2* double mutants (Figures 5D–5F), as was the case in all other brain areas examined (not shown). Taken together, these data suggest that *Olig1*

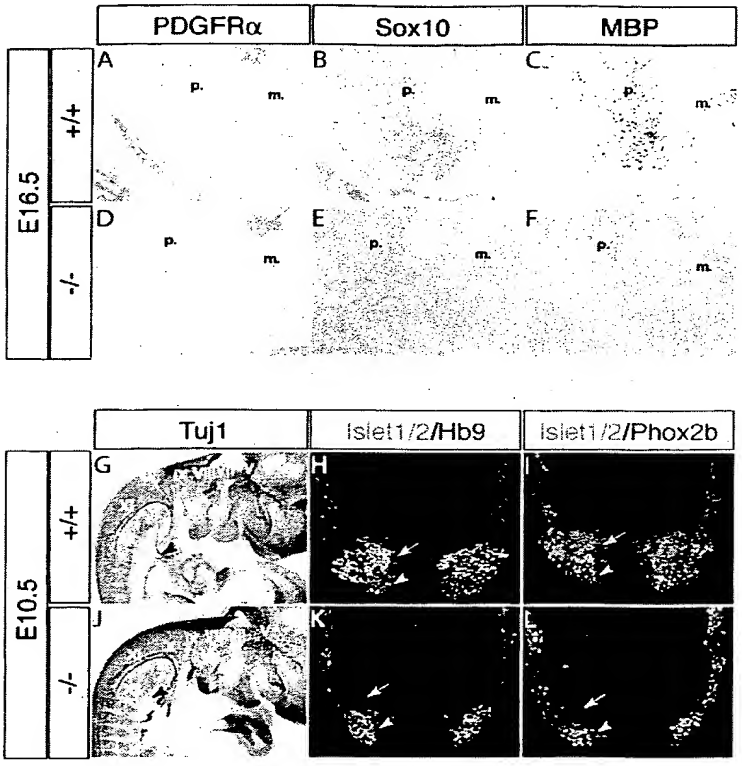

Figure 5. Loss of Hindbrain Oligodendrocytes in the Null Mutant Is Preceded by the Loss of Hindbrain Somatic But Not Visceral Motoneurons

(A–F) In situ hybridization was performed on head saggital sections of E16.5 wild-type or double-null mutant embryos. The pictures were taken from the midbrain-hindbrain region encompassing pons (p.) and medulla (m.). No oligodendrocytes were present in the hindbrain (D–F) as well as other brain areas of the double mutants (data not shown).

(G and J) Whole-mount Tuj1 antibody labeling of E10.5 embryos. The XIIth cranial motor nerve (hypoglossal, arrowhead) was missing from the homozygote.

(H–L) In the caudal hindbrain of the null mutant, Isl1/2$^+$ and Hb9$^+$ somatic motoneurons were lost (K and L, arrows), while Isl1/2$^+$ and Hb9$^-$ visceral motoneurons were still present (K and L, arrowheads).

can compensate for the lack of *Olig2* in oligodendrocyte (but not somatic motoneuron) generation in the hindbrain but not in other regions of the CNS.

Oligodendrocyte Precursors Are Transformed into Astrocytes in the Absence of *Olig2* and *Olig1*

The absence of oligodendrocyte precursors in *Olig1/2* double-mutant embryos could reflect a failure of specification, their death, or their respecification into other cell types. To distinguish between these possibilities, we first tested whether there was increased cell death in the *Olig1*$^{-/-}$*Olig2*$^{-/-}$ spinal cord from E12 to E14, a period when oligodendrocyte precursors are specified in the ventricular zone. TUNEL labeling detected no increase in apoptotic cells in either the ventricular zone or elsewhere in the spinal cord during this interval (data not shown). Next, we used the knockin marker hGFP as a short-term lineage tracer to compare the fate of *Olig2*-expressing progenitors in the presence or absence of *Olig1/2* function.

In heterozygous embryos at E13.5, individual GFP$^+$ precursors could be seen migrating away from the focus of *Olig2* expression in the ventricular zone (Figure 6A, arrow and white arrowheads). By E16.5, only a few GFP$^+$ cells remained at this focus (Figure 6B, arrow), and most had migrated into the gray matter. By E18.5, GFP$^+$ oligodendrocyte precursors were evenly distributed throughout the spinal cord, and ventricular expression was no longer detected (Figure 6C). The pattern of migration of GFP$^+$ cells in the heterozygous spinal cord closely resembles that revealed by antibody staining for endogenous OLIG2 protein (Figures 1I–1K, arrows and data not shown).

In homozygous mutant embryos, the distribution of GFP$^+$ cells was different in several respects. First, although many GFP$^+$ cells were present in the ventricular zone at E13.5 (Figure 6D, arrow), there was little migration into the gray matter. Second, the ventricular focus of GFP expression appeared larger in homozygous than in heterozygous embryos (Figures 6A versus 6D, arrows). Cell counts indicated a similar number of GFP-expressing cells in the null mutant versus heterozygous spinal cord at this stage (Figure 6G, E13.5), suggesting that *Olig2*-expressing cells may have been generated in correct numbers in the homozygote but somehow failed to migrate on schedule. At E16.5 and E18.5, however, there was a reduction in the number of GFP$^+$ cells in the double mutant (Figure 6G, white bars). This difference likely reflects reduced proliferation rather than death, as TUNEL labeling revealed no differences between the double mutant and heterozygote at these stages (data not shown).

By E16.5, although ventricular expression of GFP in homozygotes persisted (Figure 6E, arrow), GFP$^+$ cells could be seen migrating into the gray matter (Figure 6E, arrowheads). However, these cells took a more ventral trajectory than in heterozygotes. At E18.5, many GFP$^+$ cells could be detected at the pial surface of the ventral white matter in homozygotes (Figure 6F, open arrowheads), a location not occupied by GFP$^+$ cells in hetero-

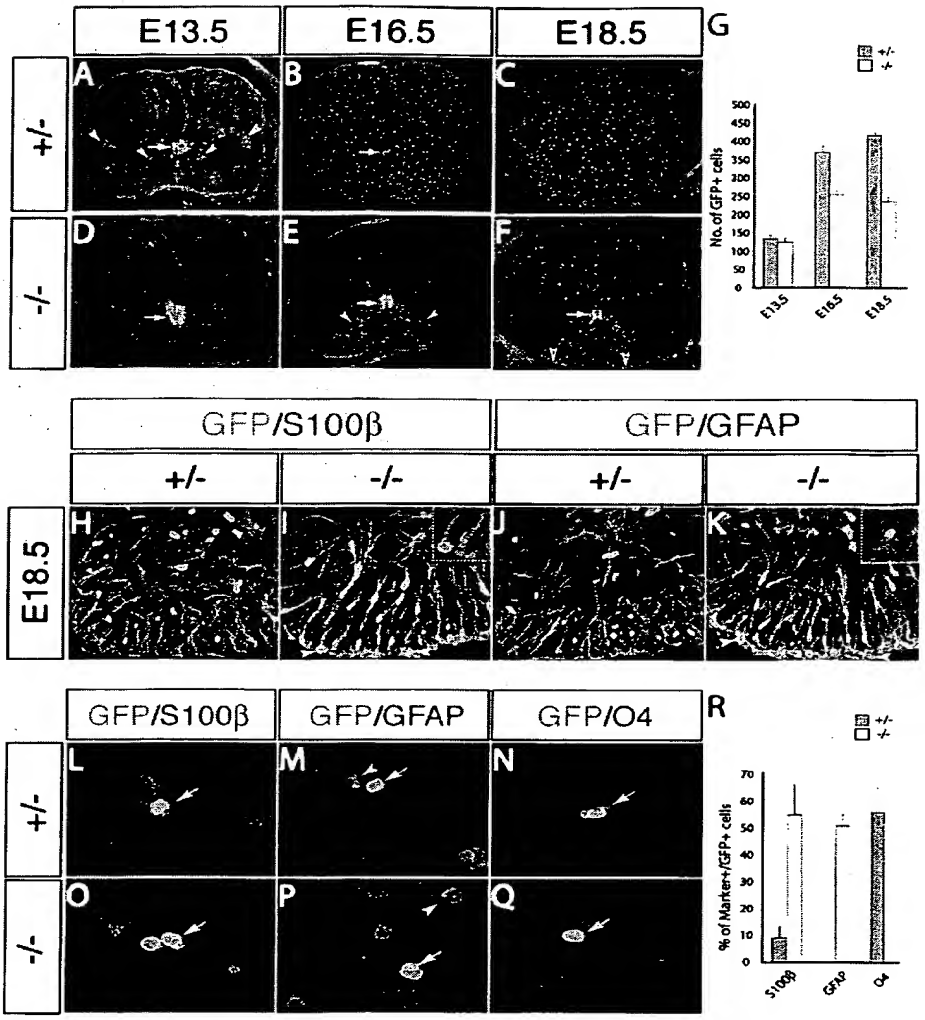

Figure 6. Oligodendrocytes Are Transformed into Astrocytes in the Absence of *Olig1* and *Olig2*

(A–G) Crosssections of heterozygous and homozygous thoracic spinal cord at indicated stages were labeled with an anti-GFP antibody. Arrows indicate ventricular expression of GFP. Persistence of GFP was apparent in both somatic (A, red arrowheads) and visceral (A, yellow arrowheads) motoneurons in the heterozygotes at E13.5, a time when the migration of GFP$^+$ oligodendrocyte precursors just started (A, white arrowheads). Open arrows in (F) indicate GFP$^+$ cells located within pial surface. Quantitative analysis (G; mean ± S.D., eight sections from two embryos) revealed a difference in the number of GFP$^+$ cells in the null mutants versus heterozygotes at E16.5 and E18.5, but not E13.5. (H–K) Many GFP$^+$ nuclei are associated closely with S100β$^+$or GFAP$^+$ fibers in the ventral spinal cord of homozygotes (I and K) but not of heterozygotes (H and J) at E18.5. Arrows point to GFP$^+$ cells in the pial surface of the null mutant spinal cord. Inserts in (I) and (K) show double-labeled cells at higher magnification.

(L–R) Staining of acutely dissociated spinal cord cells at E18.5 from either heterozygotes (L–N) or homozygotes (O–Q). Arrows point to GFP$^+$ cells. Arrowheads in (M) and (P) mark GFP$^-$GFAP$^+$ cells. Quantitative analysis (R) was performed by counting all GFP$^+$ cells from six different preps derived from different animals. Each prep contained ~5000 cells.

zygotes (Figure 6C). This observation suggested that the GFP$^+$ cells might have been transformed into astrocytes. To address this possibility, crosssections of E18.5 heterozygous or homozygous spinal cord were double labeled with antibodies to GFP and the astroglial markers GFAP or S100β. This analysis revealed that many GFP$^+$ cells in the null mutant spinal cord coexpressed GFAP or S100β (Figures 6I and 6K, arrowheads and insets). In contrast, no colocalization of GFP with either of these markers was observed in the heterozygous spinal cord (Figures 6H and 6J), consistent with previous reports that *Olig2* is not expressed in cells of the astroglial lineage in wild-type embryos (Lu et al., 2000; Zhou et al., 2000).

The filamentous staining pattern of GFAP and S100β precluded an accurate quantification of the percentage of GFP$^+$ cells that were GFAP$^+$ or S100β$^+$ in vivo. To circumvent this problem, we performed double-labeling on acutely dissociated spinal cord cells from E18.5 heterozygous and homozygous embryos. Over 50% of GFP$^+$ cells in homozygous spinal cord coexpressed GFAP (Figures 6P, arrow, and 6R, GFAP, white bar). In sharp contrast, none of the GFP$^+$ cells in the heterozygous spinal cord was found to be GFAP$^+$ (Figures 6M, arrow versus arrowhead, and 6R, gray bars). Similarly, 44% to 66% of GFP$^+$ cells were S100β$^+$ in the homozygote (Figures 6O, arrow, and 6R, white bar), whereas less than 10% of GFP$^+$ cells in the heterozygote were S100β$^+$ (Figures 6L, arrow, and 6R, gray bar). Conversely, the oligodendrocyte cell-surface marker O4 decorated over 55% of GFP$^+$ cells in the heterozygous spinal cord (Figures 6N, arrow, and 6R, gray bar) but none in the homozygote (Figures 6Q, arrow, and 6R, white bar). The reciprocal change in the percentage of GFP$^+$ cells expressing O4 versus GFAP or S100β in heterozygous versus double homozygous embryos (Figure 6R) strongly suggests that in the absence of *Olig1/2* function, the *Olig2*-expressing cell population produces astrocytes rather than oligodendrocytes. Consistent with this conclusion, in cultures of neural progenitors from *Olig1/2* homozygous embryonic spinal cord, many Olig2-hGFP-expressing cells differentiated to astrocytes but none differentiated to oligodendrocytes, whereas in cultures from heterozygotes, many GFP$^+$ oligodendrocytes developed (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1).

Discussion

The bHLH transcription factors OLIG1 and OLIG2 are sequentially expressed in motoneuron progenitors (Takebayashi et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001) and oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000). Here we show that in the absence of *Olig1* and *2* function, motoneurons are converted to V2 interneurons in the spinal cord, while oligodendrocytes fail to differentiate throughout the nervous system. Our results suggest that oligodendrocyte precursors are not simply eliminated, but instead differentiate to astrocytes. These observations are consistent with the idea that in *Olig1/2* double mutants, *Olig2*-expressing progenitors sequentially generate interneurons and astrocytes rather than motoneurons and oligodendrocytes. In this way, *Olig* genes link the specification of a particular neuronal subtype to that of a specific glial subtype, independent of the decision between neuronal versus glial fates.

Olig2 Is Required for Both the Regional Identity and Differentiation of Motoneuron Precursors

Misexpression studies in the chick have suggested that OLIG2 plays two roles in motoneuron fate determination: it specifies the regional identity of the pMN domain via repression of *Irx3*, and it promotes motoneuron progenitor cell cycle exit and differentiation, in part via local derepression of *Ngn2* (Mizuguchi et al., 2001; Novitch et al., 2001). The loss-of-function data presented in this and the companion paper (Lu et al., 2002 [this issue of *Cell*]) strengthen this view. Combined deletion of *Olig2* and *Olig1* causes a derepression of *Irx3* in pMN and a loss of *Ngn2* expression in this domain. The selective loss of *Ngn2* expression in pMN is consistent with the idea that this bHLH factor is controlled by distinct *trans*-acting factors in different progenitor domains (Scardigli et al., 2001). The motoneuron deficit in the *Olig1/2* double knockout is similar to that seen in embryos lacking *Nkx6.1/6.2* (Vallstedt et al., 2001), a homeodomain patterning molecule (Briscoe et al., 2000) that is required for *Olig2* expression (Novitch et al., 2001). The fact that expression of *Nkx6.1* and *6.2* is unperturbed in *Olig1/2* knockouts is consistent with the idea that *Olig* genes function downstream of *Nkx6.1/6.2* in motoneuron generation (Novitch et al., 2001).

Olig Genes Function Cell Autonomously in Oligodendrocyte Fate Specification

The complete failure of oligodendrocyte formation in *Olig1/2* double mutants suggests that all oligodendrocytes require *Olig* genes. Consistent with this, oligodendrocytes are not generated in neurosphere cultures derived from *Olig1*$^{-/-}$*2*$^{-/-}$ spinal cord (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/1/61/DC1). The fact that *Olig1/2* are coexpressed in oligodendrocyte precursors (this study; Lu et al., 2000; Zhou et al., 2000) suggests that this defect likely reflects a cell-autonomous requirement for these genes. An alternative explanation, however, is that this phenotype is a non-cell-autonomous consequence of the earlier loss of motoneurons, which have been hypothesized to send a feedback signal to the ventricular zone to regulate the subsequent production of oligodendrocytes (Hardy, 1997; discussed in Richardson et al., 2000).

We think this hypothesis is unlikely, however, because in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ double mutants, neuronal differentiation in the ventral spinal cord is largely eliminated (Scardigli et al., 2001), but oligodendrocyte precursor formation is unaffected (our unpublished observations). Similarly, in *Isl1*$^{-/-}$ mice which lack motoneurons (Pfaff et al., 1996), oligodendrocyte differentiation is also unaffected (Sun et al., 1998). Finally, oligodendrocytes develop normally in the hindbrain of *Olig2* single mutants (Lu et al., 2002 [this issue of *Cell*]), which lack somatic motoneurons. Our observation that in *Olig1/2* double mutants, hindbrain oligodendrocytes are completely lost further indicates that the sparing of hindbrain oligodendrocytes in *Olig2*$^{-/-}$ embryos is not due to compensation of a somatic motoneuron-derived signal by visceral motoneurons, which are spared in both *Olig2*$^{-/-}$ and *Olig1*$^{-/-}$*2*$^{-/-}$ mutants.

Role of *Olig* Genes in Motoneuron and Oligodendrocyte Fate Specification

The inference that *Olig* genes function cell autonomously in oligodendrocyte development leaves open the question of when that function is required. Our data indicate that both motoneurons and oligodendrocytes are normally generated from pMN (but not from p2) and support the idea (Richardson et al., 1997, 2000) that these neurons and glia share a common precursor. Consequently, the homeotic-like transformation of such pre-

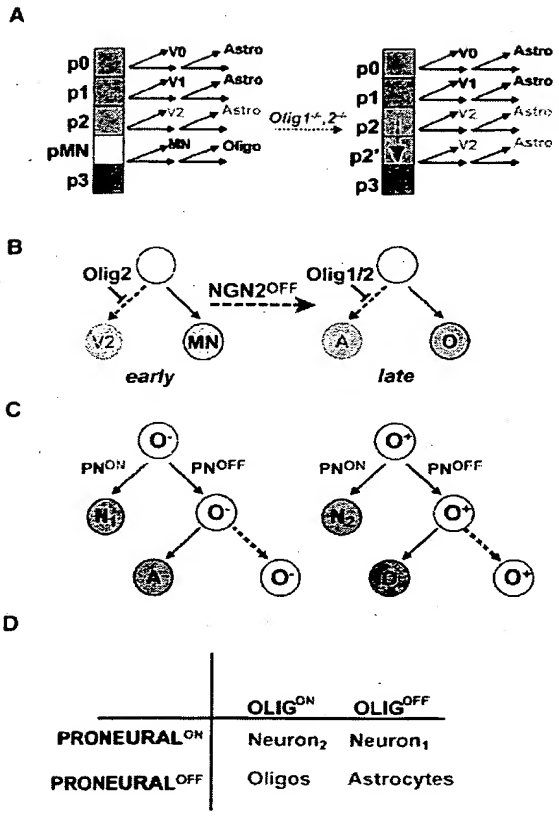

Figure 7. *Olig* Genes Control the Subtype Identities of Both Neurons and Glia Derived from a Common Progenitor Domain

(A) Summary of neuronal and glial phenotypes in *Olig1*$^{-/-}$*Olig2*$^{-/-}$ mutants.

(B) *Olig* genes may act sequentially in motoneuron (MN) and oligodendrocyte (O) development. Abbreviation: A, astrocyte.

(C) Two putative types of spinal cord progenitor cells. O$^-$ progenitors do not express *Olig* genes and generate certain types of neurons (N_1) and later astrocytes (A). Abbreviation: PN, proneural. O$^+$ progenitors first generate other types of neurons (N_2) and then oligodendrocytes (O, blue cell).

(D) A simple combinatorial code composed of proneural and *Olig* genes can determine whether CNS progenitors produce neurons, oligodendrocytes, or astrocytes. Neuron$_1$ and Neuron$_2$ denote two different neuronal subtypes.

cursors from a pMN to a p2 identity in *Olig1/2* homozygous embryos could result in the elimination of both cell types (Figure 7A). In that case, the wild-type function of *Olig* genes in specifying both the motoneuron and oligodendrocyte fates might simply be repression of *Irx3* in pMN. Alternatively, the mutant phenotype could reflect an independent and sequential requirement for *Olig* genes in both early patterning of the pMN domain and in oligodendrocyte fate determination (Figure 7B).

Consistent with the idea of independent functions, the hindbrain phenotype of *Olig2*$^{-/-}$ single mutants demonstrates that *Olig* gene mutations can cause deficiencies in somatic motoneuron generation without necessarily affecting oligodendrocyte development (Lu et al., 2002 [this issue of *Cell*]). Furthermore, while misexpression of *Olig2* in chick is sufficient to cause ectopic repression of *Irx3* and motoneuron induction in some regions of the spinal cord (Novitch et al., 2001), it does not induce ectopic oligodendrocyte differentiation (Zhou et al., 2001). Thus, while repression of *Irx3* by *Olig* genes may be necessary for oligodendrocyte fate determination, it may not be sufficient. Formal resolution of this issue will require selective rescue of the early pMN phase of *Olig2* expression in *Olig1/2* double knockouts to determine whether both motoneurons and oligodendrocytes are recovered.

OLIG1 and 2 Control an Oligodendrocyte versus Astrocyte Fate Choice

Our lineage-tracing data suggest that many *Olig2-hGFP*-expressing precursors generate astrocytes instead of oligodendrocytes upon deletion of *Olig1* and *Olig2*. If so, it would imply that spinal cord oligodendrocyte precursors have the potential to generate astrocytes, but that this fate is normally repressed by *Olig* genes (Figure 7B, right). Astrocytes are thought to arise from multiple levels along the dorso-ventral axis of the spinal cord (Pringle et al., 1998). Thus, it is a reasonable assumption that the p2 domain normally generates astrocytes after it generates V2 interneurons (Figure 7A, left). If so, then the trans-fating of *Olig2*-expressing progenitors to astrocytes in *Olig1/2* double mutants could reflect a conversion of progenitors that sequentially generate motoneurons and oligodendrocytes to ones that produce first V2 interneurons and then astrocytes (Figure 7A, right, p2').

If deletion of *Olig* genes causes oligodendrocyte precursors to generate astrocytes, do such precursors normally generate astrocytes following downregulation of *Olig* gene expression in the ventricular zone? We found no evidence for persistence of the OLIG2-hGFP lineage marker into astrocytes in *Olig1/2* heterozygotes. Similarly, using a permanent lineage tracer, Lu et al. (2002 [this issue of *Cell*]) found no astrocytes among the progeny of *Olig1*-expressing cells. These data suggest that *Olig*-expressing progenitors normally produce motoneurons and oligodendrocytes but not astrocytes in vivo (Figure 7C, O$^+$). This idea may seem inconsistent with the demonstration that single CNS progenitors can generate neurons, astrocytes, and oligodendrocytes in culture (reviewed in Gage, 2000; Anderson, 2001). However, despite extensive retroviral lineage tracing studies, there is no clear evidence for tripotential neuron/astrocyte/oligodendrocyte progenitors in vivo (Luskin et al., 1988; Leber et al., 1990). The tripotential CNS stem cells characterized in vitro may thus represent a more primitive progenitor than has been identified in vivo. Alternatively, the cell culture environment may reveal a combination of developmental potentials that are not actually used by any single progenitor in vivo.

The Genetic Logic of Neural Cell Fate Determination

bHLH proneural genes such as the *Ngns* control a neuron versus glial fate switch (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001b; reviewed in Vetter, 2001). In

the case of motoneurons and oligodendrocytes, *Ngn1/2* is likely to be the proneural gene that controls this switch (this study; Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data, taken together with our previous results (Zhou et al., 2001), suggest that once expression of *Ngns* has been extinguished in pMN, *Olig* genes determine whether the remaining progenitors will produce oligodendrocytes or astrocytes (Figure 7B).

These results suggest a simple combinatorial code whereby different combinations of the *Olig* and proneural genes can specify either neuronal, oligodendroglial, or astroglial fates (Figure 7D). According to this genetic logic, the astroglial fate would represent a final "ground state," in which neither proneural nor *Olig* genes are expressed. This fate may, however, require active repression by *Hes* genes, which repress proneural gene expression (Ishibashi et al., 1995; Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001; reviewed in Fisher and Caudy, 1998).

Combinatorial codes of Lim HD and Ets domain transcription factors have been shown to control different aspects of motoneuron subtype identity (Tsuchida et al., 1994; Lin et al., 1998; Sharma et al., 1998; Kania et al., 2000). By contrast, bHLH factors have until now been viewed as primarily acting in linear cascades to produce a single cell type, such as muscle or neuron (Weintraub, 1993; Lee, 1997). The results presented here suggest that in the nervous system, bHLH factors can also function in a combinatorial code that determines the three fundamental cell types of the CNS. This code may provide a foundation upon which higher-order aspects of neuronal and glial subtype identity can be built by superimposing combinatorial codes composed of other families of transcription factors. The linking of these multiple coding systems may then be achieved by crossregulatory, and perhaps physical, interactions between the molecules that comprise them.

Experimental Procedures

Generation of Olig1 and Olig2 Double-Mutant Mice

All mouse genomic clones were derived from a 129SVJ genomic library (Stratagene). Both the mouse *Olig1* and *Olig2* sequences are encoded by a single exon. The *Olig2* targeting vector was constructed by inserting a *Histone-GFP fusion/loxp/PGKneo* cassette between a 2 kb 5′ arm and a 3.8 kb 3′ arm. For the *Olig1* targeting vector, a *tau LacZ/loxp/PGKhyg* cassette was cloned between the 1.9 kb 5′ arm and the 3.2 kb 3′ arm.

Two rounds of electroporation and selection were conducted, first with the *Olig2* targeting vector, and then with the *Olig1* targeting vector. Correctly recombined clones at both the *Olig2* and *Olig1* loci were subjected to Cre/Loxp analysis to determine whether the two recombined alleles reside on the same chromosome (protocol available upon request). The frequency of recombination for both the *Olig2* and *Olig1* loci was ~1:300. Clones in which the two mutant alleles are located in *cis* were injected into C57BL/6J blastocysts to generate germline chimeric founders. Mutant mice were genotyped with PCR primers specific to *Olig1*, *Olig2*, *GFP*, *lacZ*, *Neomycin*, and *Hygromycin* genes. No segregation of the two mutant alleles has been observed in all embryos genotyped so far. All embryos analyzed in this study were derived from heterozygous 129sv × C57BL/6J intercrosses.

In Situ Hybridization

Nonradioactive in situ hybridization was performed as previously described (Zhou et al., 2000). The following mouse gene probes were used: *Olig1*, *Olig2*, *Olig3*, *Sox10* (a gift of Dr. Kirsten Kuhlbrodt), *PDGFR*, *MBP*, *PLP/DM20*, *Ngn1*, and *Ngn3*. Probes for *Nkx6.1*, *Nkx6.2*, and *Dbx2* were the kind gift of Dr. Thomas Jessell.

Immunohistochemistry

Mouse embryos were fixed by immersion in 4% paraformaldehyde from 1 hr to overnight at 4°C depending on the age. The following primary antibodies were used: rabbit anti-Olig2 (1:2000, gift of Dr. Takebayashi Hirohide), rabbit anti-Nkx2.2 (1:1000, gift of Dr. Thomas Jessell), mAb anti-Neurogenin2 (1:100, Liching Lo), rabbit anti-Chx10 (1:4000, gift of Dr. Thomas Jessell), guinea pig anti-Irx3 (1:1000, gift of Dr. Thomas Jessell), rabbit anti-Hb9 (1:2000, gift of Dr. Samuel Pfaff), rabbit anti-GFP and Alexa-488 conjugated rabbit anti-GFP (1:1000, Molecular Probes), rabbit anti β-gal (1:1000, 5′-3′), rabbit anti-GFAP (1:1000, DAKO), mAb anti-S100 β (1:1000, Sigma), and rabbit anti-Phox2b (1:700, gift of Dr. Jean-Francois Brunet). mAbs against Lim3, MNR2/Hb9, Engrailed-1, Isl1/2, Hb9, Lim3, Nkx2.2, and Pax6 were obtained from Developmental Studies Hybridoma Bank (DSHB). Whole-mount antibody staining of mouse embryos was performed as described previously (Ma et al., 1996).

BrdU Labeling and TUNEL Assay

BrdU labeling of mouse embryos was conducted by intraperitoneal injection of BrdU (Sigma, 65 mg/g body weight) 2 hr before sacrifice. A rat anti-BrdU antibody (Accurate) was used to detect BrdU. TUNEL assays were performed with a kit from Roche according to the manufacturer's instructions.

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Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain

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Summary

Studies have indicated that oligodendrocytes in the spinal cord originate from a ventral progenitor domain defined by expression of the oligodendrocyte-determining bHLH proteins Olig1 and Olig2. Here, we provide evidence that progenitors in the dorsal spinal cord and hindbrain also produce oligodendrocytes and that the specification of these cells may result from a dorsal evasion of BMP signaling over time. Moreover, we show that the generation of ventral oligodendrocytes in the spinal cord depends on Nkx6.1 and Nkx6.2 function, while these homeodomain proteins in the anterior hindbrain instead suppress oligodendrocyte specification. The opposing roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, appear to reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Based on these findings, we propose that oligodendrocytes derive from several distinct positional origins and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Introduction

Neurons, oligodendrocytes, and astrocytes represent the three fundamental cell types of the vertebrate central nervous system (CNS), and the generation of these cell types at precise positions and specific time points during development is critical for the establishment of brain function. Insight has been obtained into the molecular mechanisms that control the generation of specific neuronal subtypes in space and time (Jessell, 2000; Lumsden and Krumlauf, 1996; Pattyn et al., 2003b). Oligodendrocytes and astrocytes are generated subsequent to neurogenesis (Rowitch, 2004), and less is known about the positional determination of these glial cell types during CNS development.

Oligodendrocytes are the myelinating cells of the CNS that insulate axons, while astrocytes provide structural support, regulate water balance, and maintain the blood-brain barrier (Rowitch, 2004). Glial cells originate from neural progenitors in the ventricular zone (VZ), and once specified they leave the VZ and migrate as proliferative precursors to occupy all regions of the CNS. Studies in the spinal cord suggest that oligodendrocytes are produced by a small group of ventral progenitors close to the floor plate, while astrocytes appear to be generated from more dorsally located progenitors (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). Functional analysis of the oligodendrocyte-determining basic-helix-loop-helix (bHLH) proteins Olig1 and Olig2 (collectively termed Olig1/2) support the idea that oligodendrocytes and astrocytes are generated from distinct progenitor domains and suggest further that oligodendrocytes and astrocytes are positionally specified (Lu et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2001) according to strategies similar to those determining neuronal subtypes (Jessell, 2000).

In the spinal cord, different neurons emerge at specific dorsoventral (DV) positions in response to local Sonic hedgehog (Shh) signaling by ventral midline cells (Jessell, 2000) and bone morphogenetic proteins (BMPs) secreted from the dorsal midline of the neural tube (Lee et al., 2000; Liem et al., 1997). In ventral positions, graded Shh signaling controls patterning by regulating the regional expression of a set of homeodomain (HD)-containing transcriptional repressors (Briscoe et al., 2000; Muhr et al., 2001), thereby establishing a combinatorial code of HD protein expression, which defines five progenitor domains. Each domain, in turn, produces a distinct neuronal subtype (Jessell, 2000). Olig1/2 is induced by Shh, and its expression is confined to an individual ventral progenitor domain (termed pMN domain) that sequentially produces spinal motor neurons (sMNs) and oligodendrocytes (Rowitch, 2004). In *Olig1/2* mutant mice, pMN progenitors acquire an identity typical of more dorsal progenitors, and the loss of sMNs and oligodendrocytes in these mice is accompanied by a concomitant gain of V2 neurons and astrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Apart from revealing an absolute requirement for Olig1/2 for oligodendrocyte generation, these data show that Olig1/2 also suppress astroglial fate in pMN progenitors (Zhou and Anderson, 2002), indicating that oligodendroglial and astroglial lineages are spatially separated in vivo at early developmental stages (reviewed in Rowitch, 2004).

While the data above demonstrate a restricted ventral origin of oligodendrocytes from pMN progenitors, it remains unclear if also other progenitors produce oligodendrocytes in vivo. In a series of quail-to-chick grafting experiments, Cameron-Curry and LeDouarin provided data suggesting that dorsal progenitors can produce oligodendrocytes (Cameron-Curry and Le Douarin, 1995). Similar experiments by Pringle and coworkers, however, instead argued that dorsal progenitors only generate astrocytes (Pringle et al., 1998). In vitro assays have further suggested the existence of a glial-restricted progenitor cell that can be derived from both dorsal and ventral parts of the spinal cord and give rise to oligodendrocytes and astrocytes in culture (Rao et al., 1998). Also, tripotent self-renewing stem cells that generate neurons, oligodendrocytes, and astrocytes in vitro can be isolated from most parts of the developing and adult CNS (Gage, 2000; Qian et al., 2000; Seaberg and van der Kooy, 2003), implicating that oligodendrocytes and astrocytes are derived from common precursors

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broadly distributed in the CNS. However, a recent study has questioned the presence of such tripotent stem cells in vivo, since the exposure of cells to fibroblast growth factor 2 (FGF2), the primary mitogen used to propagate stem cells, can result in a deregulated positional identity of neural progenitors in vitro (Gabay et al., 2003). The potency of progenitors isolated from a given position of the CNS may therefore reflect a quality acquired from exposure to FGF2 in vitro, rather than revealing their endogenous capacity in vivo. Thus, while it is well established that oligodendrocytes are produced by ventral progenitors, it is uncertain if also other regions of the developing CNS give rise to these cell types in vivo.

We have explored the positional specification of oligodendrocytes in the spinal cord and hindbrain and provide in vivo and in vitro evidence that, in addition to the ventral pMN domain, oligodendrocytes are generated from dorsal progenitors at both these axial levels. High concentrations of BMPs block the specification of dorsal Olig2$^+$ cells in vitro, and their generation is promoted when BMP signaling is inhibited, indicating that a progressive decrease of dorsal BMP signaling over time influences the temporal appearance of oligodendrocytes in the dorsal neural tube. In addition, we show that pMN domain-derived oligodendrocytes essentially are missing in the spinal cord of mice lacking the ventrally expressed HD proteins Nkx6.1 and Nkx6.2 (collectively termed Nkx6 proteins). We find that these HD proteins instead suppress oligodendrocyte production in the anterior hindbrain. These unanticipated opposite roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Taken together, our data suggest that oligodendrocytes are generated from several distinct DV progenitor domains and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Results

A Dorsal Origin of Oligodendrocyte Generation in the Hindbrain

Similar to the spinal cord, oligodendrocytes in the hindbrain are generated from ventral progenitors, and their production depends on Shh signaling (Alberta et al., 2001) and Olig1/2 function (Lu et al., 2002; Zhou and Anderson, 2002). However, Olig1/2 expression in the hindbrain is also detected in the VZ at more dorsal positions (Figures 1A and 1B) (Liu et al., 2003), and the fate of these cells has not been determined. At E13.5, one dorsal *Olig1/2* expression domain spans along the axial extent of the hindbrain, with the exception of rhombomere 1 (Figure 1A). To begin to examine this population of Olig1/2$^+$ cells, we mapped their DV position in relation to the expression of HD proteins that define different DV progenitor domains. While ventral *Olig1*$^+$ cells at E13.5 were detected within the pMNv domain that expresses the HD protein *Nkx2.2* (see below), the dorsal Olig1$^+$ cells were located immediately dorsal to the expression domain of *Dbx2* (Pierani et al., 1999), but within the domain of *Pax3*, *Pax7*, and *Gsh1* expression (Figures 1B–1F; data not shown). Pax3, Pax7, and Gsh1 are definitive markers of dorsal progenitor cells at this axial level (Goulding et al., 1991; Jostes et al., 1990; Sander et al., 2000; Valerius et al., 1995), suggesting that this population of Olig1/2$^+$ cells is located within the alar plate. The OLP marker *Sox10* (Kuhlbrodt et al., 1998) was expressed in a fashion similar to *Olig1* in the dorsal hindbrain (Figure 1G). As determined by immunohistochemistry, several dorsal Olig2$^+$ cells in the VZ coexpressed Gsh1 (Figure 1H) while more laterally positioned, and presumably more mature, cells coexpressed the OLP markers PDGFRα (Hall et al., 1996) and NG2 (Figures 1J and 1K) (Nishiyama et al., 1996) but not the panneuronal marker NeuN (Figure 1I) (Mullen et al., 1992). Together, these data strongly suggest that at least a subset of Olig1/2$^+$ cells in the hindbrain originates from dorsal progenitors and are consistent with the idea that these cells differentiate into oligodendrocytes.

To more extensively investigate if dorsal progenitors in the hindbrain generate oligodendrocytes, we examined the capacity of hindbrain explants to generate OLPs in vitro. In this assay, explants corresponding to the ventral and dorsal Olig1/2$^+$ domains (Figure 1X) were isolated at E10.5, approximately 2–3 days before Olig1/2$^+$ OLPs can be detected in vivo (Miller, 2002). Explants isolated from tissue intervening the ventral and dorsal Olig1/2$^+$ domains (intermediate explants; Figure 1X) were included as controls, since this Olig1/2$^-$ domain is predicted not to produce oligodendrocytes in vivo or in vitro. Explants were cultured in defined media containing platelet-derived growth factor (PDGF-AA) for various time points, and OLP generation was scored by monitoring expression of the OLP markers Olig2, PDGFRα, NG2, and the O4 antigen (Sommer and Schachner, 1981). Importantly, we did not add fibroblast growth factors (FGFs) to the culture media, since FGF2 has been shown to ventralize cultured dorsal neural progenitor cells, resulting in an arbitrary in vitro-triggered induction of Olig2 expression and oligodendrocyte differentiation (Chandran et al., 2003; Gabay et al., 2003).

In ventral and dorsal explants cultured for 6 days, an extensive number of Olig2$^+$ cells were detected, and the majority of cells coexpressed PDGFRα and NG2 (Figures 1L, 1N, 1O, and 1Q). After 8–10 days in vitro, ventral explants showed significant expression of the more mature oligodendrocyte lineage marker O4 (Figure 1T). Olig2$^+$/O4$^+$ cells were detected also in dorsal explants, albeit the number of double-positive cells was lower as compared to ventral explants (Figure 1R). Importantly, in intermediate explants cultured under identical conditions, expression of Olig2, PDGFRα, or O4 was not detected at any time point analyzed (3–10 days of culture; Figures 1M, 1P, and 1S and data not shown). Thus, progenitors isolated from a DV domain that lacks expression of Olig1/2 in vivo do not generate OLPs under these in vitro culturing conditions. Given that dorsal but not the more ventral intermediate explants generate OLPs, it is unlikely that the OLPs observed in dorsal explants represent ventrally derived OLPs that at the time of tissue isolation had migrated into dorsal positions. Moreover, the absence of OLPs in intermediate explants makes it unlikely that the generation of OLPs in dorsal explants result from a deregulated, or ventralized, potential of dorsal progenitors due to the culturing conditions. In additional support for this, we could detect expression of Pax7 (Figure 1U) but not the ventral mark-

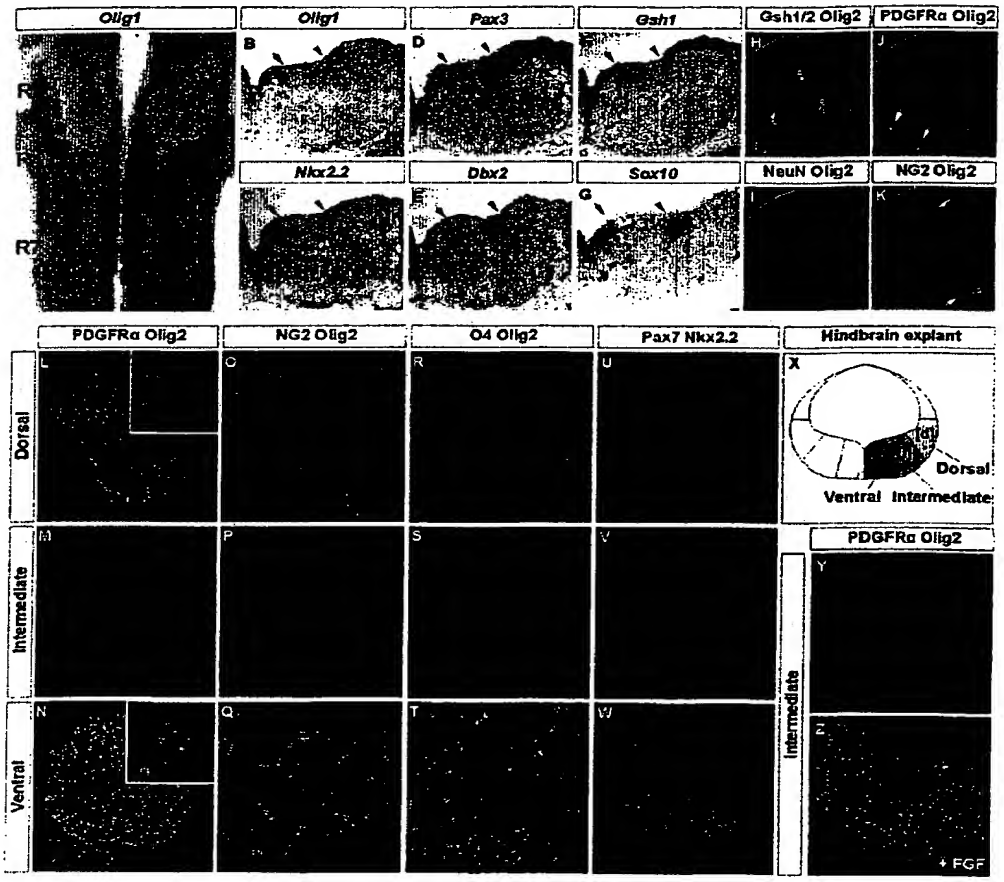

Figure 1. Dorsal Olig1/2$^+$ Progenitors in Hindbrain Give Rise to Oligodendrocytes

(A) Dorsal flat-mount view showing expression of *Olig1* in the ventral (arrow) and dorsal (arrowhead) hindbrain (HB).

(B–G) Transverse sections of rhombomere (R) 4 of the HB at E13.5 showing expression of *Olig1* (B), *Nkx2.2* (C), *Pax3* (D), *Dbx2* (E), and *Gsh1* (F). Dorsal *Olig1*$^+$ cells (ventral boundary indicated by arrowhead) are located within the domain of *Gsh1* and *Pax3*. Ventral *Olig1*$^+$ cells are detected within the *Nkx2.2*$^+$ domain (dorsal boundary indicated by arrow). *Sox10* (G) is at E12.5 expressed in a fashion similar to *Olig1* (B).

(H–K) A subset of dorsal Olig2$^+$ cells expresses Gsh1/2 (H), PDGFRα (arrows in [J]), and NG2 (arrows in [K]) but not NeuN (I).

(L–W) Ventral ([v]) and dorsal ([d]) but not intermediate ([i]) HB explants isolated at E10.5 generate oligodendrocytes in vitro. After 6 days in culture, Olig2$^+$/PDGFRα$^+$ cells are present in dorsal (inset in [L]) and ventral (inset in [N]) explants. After 8 days, Olig2$^+$ cells in ventral and dorsal explants express NG2 (O and Q) and O4 (R and T). No cells in intermediate explants expressed Olig2, PDGFRα, NG2, or O4 (M, P, and S). HB explants retain their dorsoventral identity, shown at 3 days in culture. Dorsal explants express Pax7 (U), while ventral express Nkx2.2 (W).

(X) Illustration of isolated dorsal, intermediate, and ventral explants.

(Y and Z) FGF2 induces Olig2 and PDGFRα expression in intermediate explants, shown after 8 days in culture.

ers Nkx2.2 or Shh (Figures 1U and 1W; data not shown) in dorsal explants cultured for 2–5 days.

It is possible that the absence of OLPs in intermediate explants could reflect that intermediate progenitors, in contrast to their more ventral or dorsal counterparts, have lost competence to generate oligodendrocytes at the time of tissue isolation. To examine this, we cultured intermediate explants in media enriched with FGF2. In contrast to explants cultured in PDGF-AA-enriched media without FGF2 (Figure 1Y), numerous Olig2$^+$/PDGFRα$^+$-expressing cells were detected in intermediate explants cultured in the presence of FGF2 (Figure 1Z). These data show that intermediate progenitors have the potential to produce OLPs and support the notion that addition of FGF2 to neural progenitor, or stem cell, cultures in vitro induces oligodendrocyte differentiation in cells not fated to generate these cells in vivo. Taken together, these data lend strong support to the idea that oligodendrocytes are derived from both ventral and dorsal Olig1/2$^+$ progenitor domains in the hindbrain.

Dorsal Progenitors in the Spinal Cord Generate Olig2-Expressing Cells and Give Rise to Oligodendrocytes in Culture

The generation of oligodendrocytes has been most extensively studied in the spinal cord (Rowitch, 2004). A

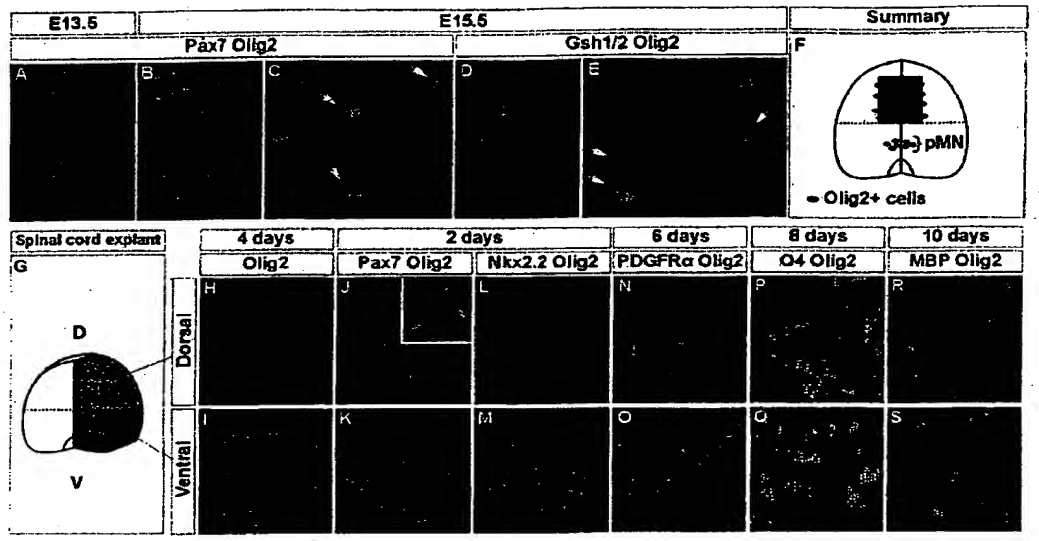

Figure 2. Dorsal Spinal Cord Progenitors Express Olig2 and Generate Oligodendrocytes In Vitro

(A–E) Transverse sections of thoracic spinal cord (SC) at E13.5 and E15.5 showing expression of Olig2 relative to Pax7 (A–C) and Gsh1/2 (D and E). Several dorsally positioned Olig2$^+$ cells coexpress Pax7 (C) and Gsh1/2 (E) at E15.5.

(F) Summary illustrating that Olig2$^+$ cells can be detected both in the ventral and dorsal ventricular zone (VZ).

(G) Illustration of the division of SC into dorsal and ventral explants.

(H–S) Whole-mount staining of dorsal and ventral explants isolated at E12.5. Cells in dorsal and ventral explants express Olig2 (H and I) and retain their dorsoventral identity after 2 days of culture; dorsal explants express Pax7 (J), and ventral explants express Nkx2.2 (M). Inset in (J) shows Olig2$^+$ cells that express Pax7. Olig2$^-$ cells in dorsal and ventral explants express PDGFRα after 6 days (N and O), O4 after 8 days (P and Q), and MBP after 10 days in vitro (R and S).

major source of oligodendrocyte production at this level is the ventral pMN domain that expresses Olig1 and Olig2 (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). It remains uncertain if also other progenitor domains in the spinal cord generate oligodendrocytes in vivo (Cameron-Curry and Le Douarin, 1995; Miller, 2002; Pringle et al., 1998; Richardson et al., 2000; Spassky et al., 2000). Our data suggesting a dual origin of oligodendrocytes in the hindbrain prompted us to examine the positional generation of oligodendrocytes in the spinal cord. In agreement with previous studies, the expression of Olig2 within the VZ was selectively confined to the ventral pMN domain at the peak of OLP specification at E13.5 (Figure 2A). At E15.5, migrating Olig2$^+$ OLPs were detected throughout the spinal cord (Figures 2B and 2D; data not shown). Interestingly, many Olig2$^+$ cells located within or in close proximity to the dorsal VZ coexpressed the dorsal progenitor markers Pax7 and Gsh1/2 at E15.5 (Figures 2C and 2E). Dorsal Olig2$^+$ cells located at a distance from the VZ did not express these markers (Figures 2B and 2D).

The presence of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ in the dorsal spinal cord raised the possibility that dorsal progenitors generate Olig2$^+$ oligodendrocytes at this level. Alternatively, a subset of migrating Olig2$^+$ cells generated from the pMN domain could invade the dorsal VZ and initiate expression of Pax7 and Gsh1/2 at E15. To distinguish between these possibilities, we compared the ability of isolated ventral and dorsal spinal cord explants to generate oligodendrocytes in vitro. In these experiments, ventral and dorsal explants were isolated at E10.5 or at E12 and thus prior to any dorsal migration of pMN-derived OLPs (Sussman et al., 2000). Explants were cultured in media containing PDGF-AA but not FGF2 for various time points. In these conditions, OLP differentiation was observed in both ventral and dorsal explants after 4–8 days of culture, as determined by Olig2$^+$ cells that coexpressed PDGFRα, NG2, and the O4 antigen (Figures 2N–2Q; data not shown). After 8–10 days, Olig2-expressing cells in ventral and dorsal explants had initiated expression of myelin basic protein (MBP), a marker of mature oligodendrocytes (Figures 2R and 2S) (Lemke, 1988). Similar results were obtained from ventral and dorsal spinal cord explants isolated from E10.5 and E12.5 embryos (data not shown).

We next examined the DV identity of cells in spinal cord dorsal and ventral explants. In dorsal explants isolated at E12, Olig2$^+$ cells could first be detected after 2–3 days of culture, a time point that corresponds well with the appearance of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ cells at around E15 in vivo (Figures 2J, 2L, and 2B–2E; data not shown). At these stages, Pax7 expression was observed while no expression of ventral markers Nkx2.2, Nkx6.1, or Shh could be detected (Figures 2J and 2L; data not shown). Importantly, several Olig2$^+$ cells in dorsal explants coexpressed Pax7 (Figure 2J). Similar results were obtained when dorsal explants were isolated at E10.5 (data not shown). In ventral explants, expression of Nkx2.2, Nkx6.1, and Shh but not Pax7 could be de-

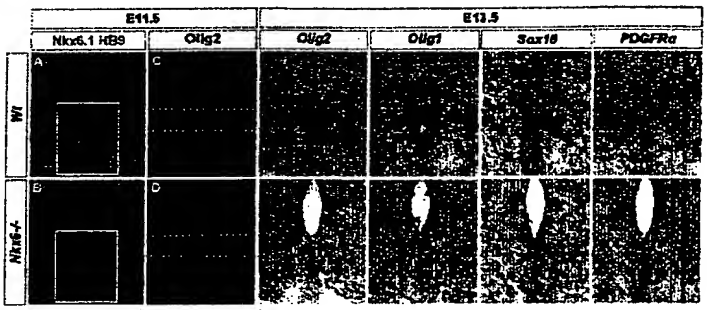

Figure 3. A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic SC sections of wild-type (wt) and *Nkx6* mutant mice at E11.5 and E13.5. Expression of Nkx6.1 and the MN marker Hb9 in wt (A) and *Nkx6* mutants (B) at E11.5. Box in (A) and (B) marks part of SC shown in (C)–(L). The expression of Olig2 in the pMN domain is lost (C and D). At E13.5, expression of the OLP markers Olig2 (E and F), *Olig1* (G and H), *Sox10* (I and J), and *PDGFRα* (K and L) are missing in the ventral spinal cord of *Nkx6* mutant mice. Dotted lines in (C)–(L) indicate pMN domain boundaries.

tected (Figures 2K and 2M; data not shown). These data show that dorsal Pax7$^+$ progenitors generate Olig2-expressing cells and that Olig2$^+$ cells differentiate along the OLP lineage in vitro and argue against the possibility that Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ detected in vivo would represent dorsally migrating cells that originate from the pMN domain. The retained DV identity of cells in dorsal explants also seems to exclude the possibility that OLPs in dorsal explants are generated in response to an in vitro-induced, albeit FGF-independent, ventralization of progenitor cell identity.

A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

To further examine the possibility of a dual ventral and dorsal origin of oligodendrocytes in the spinal cord, we analyzed mice lacking the related HD proteins Nkx6.1 and Nkx6.2. Nkx6 proteins are expressed in the ventral neural tube, including the pMN domain, and their function is necessary for the ventral expression of Olig2 and the generation of MNs in the spinal cord (Figures 3A and 3B) (Novitch et al., 2001; Vallstedt et al., 2001). The generation of oligodendrocytes in the spinal cord has not been examined in *Nkx6* mutants, but the ventral extinction of Olig2 expression raised the possibility that pMN domain-derived oligodendrocytes may be affected. In agreement with this, we could not detect any expression of *Olig1* or Olig2 in the ventral spinal cord between E11.5 and E13.5 (Figures 3D, 3F, and 3H), the time at which ventral oligodendrocytes are being specified (Hall et al., 1996). Also, while *Sox10* and *PGDFRα* could be detected in the pMN domain and/or in migrating OLPs in controls at E13.5, the expression of these OLP markers was missing in *Nkx6* mutants (Figures 3I–3L). These data show that Nkx6 proteins are required not only for the generation of spinal MNs, but also for the subsequent specification of oligodendrocytes from the pMN domain.

Oligodendrocytes Are Generated from Progenitors with a Dorsal Identity in the Spinal Cord of *Nkx6* Mutants

Olig1/2 is required for the generation of all oligodendrocytes regardless of their developmental origin in the CNS (Lu et al., 2002; Zhou and Anderson, 2002). Our data indicate that Nkx6 proteins are necessary for the generation of pMN domain-derived oligodendrocytes in the spinal cord. However, since Nkx6.1 and Nkx6.2 are expressed only in ventral progenitors, they are not predicted to affect the generation of putative dorsally derived oligodendrocytes. We therefore examined the generation of oligodendrocytes in *Nkx6* mutants at E15.5, a stage when Olig2$^+$ cells that coexpress Pax7 and Gsh1/2 can be detected in the dorsal spinal cord in wild-type embryos (Figures 2C and 2E). In controls at this stage, OLPs were evenly distributed in the spinal cord gray matter, as determined by *Olig1* expression (Figure 4C). Strikingly, numerous *Olig1*-expressing cells were observed also in *Nkx6* mutants, but in contrast to controls, essentially all *Olig1*$^+$ cells were located in the dorsal half of the spinal cord (Figure 4D). The number and distribution of Olig2$^+$ cells coexpressing Pax7 and/or Gsh1/2 in lateral positions of the dorsal VZ was similar in mutants and controls at E15 (Figures 4E–4J and 4W). These data provide strong genetic evidence that Olig1/2$^+$ cells are generated from the dorsal VZ in vivo. A few Olig1/2$^+$cells could occasionally be detected in ventral positions at E15 (Figure 4F). Therefore, we cannot exclude the possibility that a subset of Olig1/2$^+$ cells in *Nkx6* mutants are generated also from ventral progenitors.

In control embryos at E15, a subset of Olig2$^+$ cells in the dorsal spinal cord expressed PDGFRα, while only rare Olig2$^+$/PDGFRα$^+$ cells could be detected in *Nkx6* mutants at this stage (Figures 4K and 4L). We noticed that a population of Olig2$^+$ cells that lacked the expression of PDGFRα and NG2 was present at E15.5 in wild-type mice, and this population corresponded in number to the Olig2+/PDGFRα$^-$/NG2$^-$ cells observed in mutant mice (Figure 4X; data not shown). At E18.5, Olig2$^+$ cells were evenly distributed in *Nkx6* mutants (Figure 4N), and the majority of these cells had at this stage initiated expression of PDGFRα and NG2 (Figures 4P, 4R, and 4X). Consistent with the generation of oligodendrocytes from dorsal progenitors in vitro, these data provide genetic evidence that Olig1/2$^+$ cells with a dorsal origin acquire molecular characteristics of OLPs also in vivo. Additionally, the uniform distribution of Olig1/2 cells in *Nkx6* mutants at E18.5 indicates that dorsally derived OLPs migrate into the ventral neural tube, at least in conditions when the generation of pMN domain-derived oligodendrocytes is compromised. We could not examine if OLPs in *Nkx6* mutants acquire a terminal oligodendrocyte phenotype in vivo, since *Nkx6* mutant embryos die at birth, the time when OLPs begin to terminally

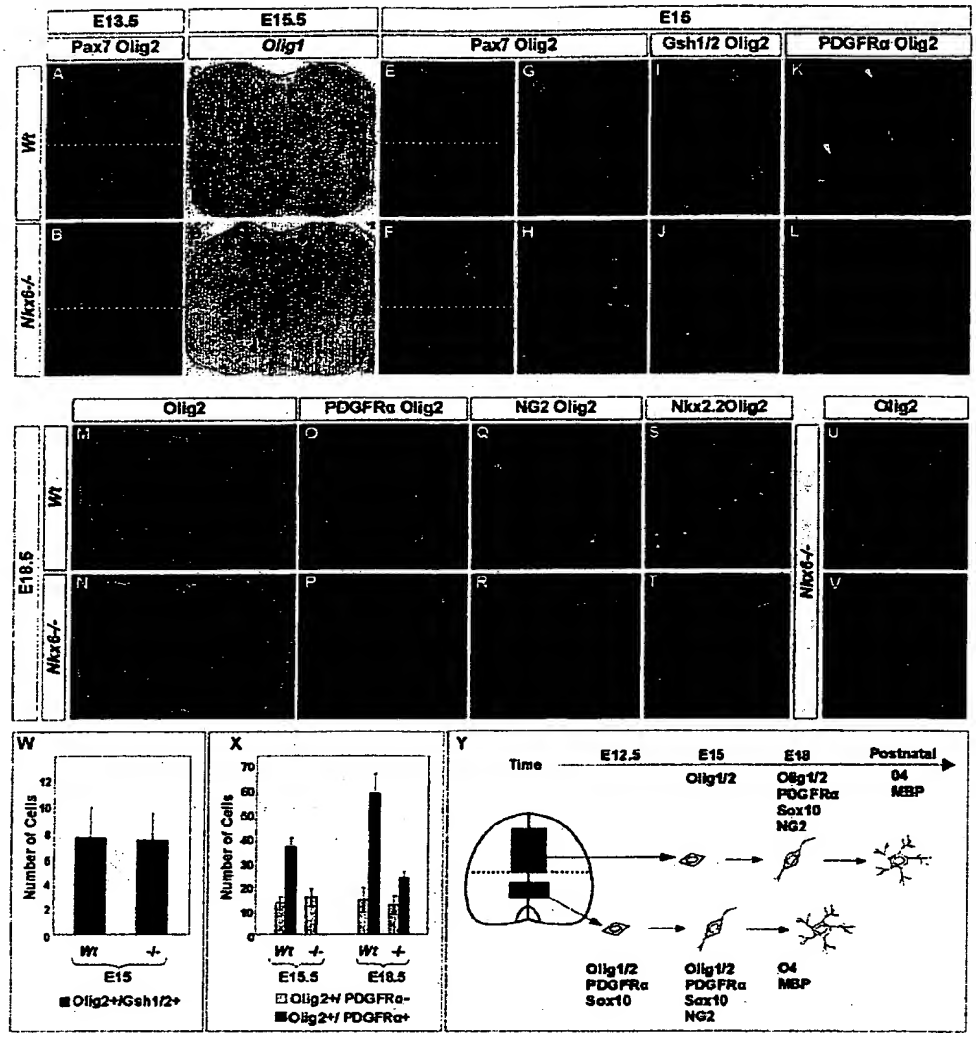

Figure 4. Dorsal Olig1/2$^+$ Cells Generate Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic SC sections of wt and *Nkx6* mutant embryos at E13.5 to E15.5. Ventral Olig2$^+$ cells are detected in controls (A) but not *Nkx6* mutants (B) at E13.5. At E15.5, *Olig1* expression is detected throughout the SC of controls (C). In *Nkx6* mutants, *Olig1* expression at E15.5 is detected predominantly in the dorsal SC (D). Dorsal Olig2$^+$ cells in controls and *Nkx6* mutants at E15.5 coexpress Pax7 (E–H) and Gsh1/2 (I and J). At E15.5, a small number of Olig2$^+$ cells (one to two cells per section) was also detected within, or in close proximity to, the ventral VZ in *Nkx6* mutants (F). Dotted line in (A)–(F) indicates the ventral boundary of Pax7 expression. Olig2$^+$ cells in *Nkx6* mutants do not express PDGFRα at E15.5 (L), while both Olig2$^+$/PDGFRα$^-$ cells (arrow in [K]) and Olig2$^+$/PDGFRα$^+$ cells (arrowhead in [K]) are found in controls at this stage.

(M–T) Transverse thoracic SC sections of wt and *Nkx6* mutant mice at E18.5. Olig2$^+$ cells are evenly distributed along the DV axis in wt (M) and *Nkx6* mutants (N), and the majority of cells express PDGFRα (O and P) and NG2 (Q and R). Olig2$^+$/Nkx2.2$^+$ are detected in wt (S) but not *Nkx6* mutant embryos (T).

(U and V) *Nkx6* mutant SC tissue isolated at E12.5 and cultured for 10 days generate Olig2$^+$ cells that coexpress O4 (U) and MBP (V).

(W) Quantification of Olig2$^+$/Gsh1/2$^+$ cells in wt and *Nkx6* mutants at E15.5. Counts from six to eight sections per embryo; n = 2 wt and 3 *Nkx6* mutants; mean ± SD.

(X) Quantification of Olig2$^+$/PDGFRα$^-$ and Olig2$^+$/PDGFRα$^+$ in the SC of wt and *Nkx6* mutant embryos at E15.5 and E18.5. Similar numbers of Olig2$^+$/PDGFRα$^-$ are present in wt and mutant mice at both time points. Counts from ten sections; mean ± SD.

(Y) Model indicating the differentiation profile of ventral and dorsal oligodendrocytes in the SC.

differentiate (Baumann and Pham-Dinh, 2001). Nevertheless, Olig2$^+$ cells that expressed O4 and MBP could be detected in isolated *Nkx6* mutant spinal cord tissue after culture in vitro (Figures 4U and 4V), indicating that these cells have the capacity to differentiate into mature oligodendrocytes.

Dorsal and Ventral OLP Lineages Express Distinct Molecular Properties at Prenatal Stages

Are oligodendrocytes generated from distinct positional origins molecularly and functionally equivalent? Studies of neuronal differentiation suggest that neurons with similar functional properties have certain common molecular properties, but also that functional differences among cells within a given class are associated with subtype-specific profiles of gene expression (Jessell, 2000). As compared to neuronal cell differentiation, little is known about the determination of different glial subtypes (Rowitch, 2004). To examine the molecular properties of dorsally and ventrally derived oligodendrocytes, we compared the gene expression profile of Olig2$^+$ OLPs in controls and *Nkx6* mutant mice at E18.5. In controls, a subset of Olig2$^+$/PDGFRα$^+$ cells in the spinal cord had initiated expression of Nkx2.2 (29% ± 2.5%) (Figure 4S) (Qi et al., 2001). In contrast, few if any Olig2$^+$/PDGFRα$^+$ cells in *Nkx6* mutants expressed Nkx2.2 at this stage (Figure 4T). The lack of OLPs that express Nkx2.2 in *Nkx6* mutants indicate that Nkx2.2 selectively marks OLPs with a ventral origin and that OLPs with different origins are molecularly distinct, at least at prenatal stages of spinal cord development.

Evasion of BMP Signals Influences the Timing of Olig1/2 Induction in Dorsal Progenitors

The occurrence of dorsally generated oligodendrocytes raises the issue of how these cells are specified. In the neural tube, local BMP signaling has a central role in the initial establishment of dorsal progenitor identity (Lee et al., 2000; Liem et al., 1997; Nguyen et al., 2000). BMPs expressed in the dorsal neural tube also function to suppress more ventral Shh-dependent cell fates, including MNs (Liem et al., 2000) and ventral oligodendrocytes derived from the pMN domain (Hall and Miller, 2004; Mekki-Dauriac et al., 2002). The activation of Olig1/2 expression in dorsal progenitors occurs at around E15, a stage when the neural tube has grown considerably in size. We therefore considered the possibility that dorsally generated oligodendrocytes could be sensitive to BMPs and that their late birth could reflect a decrease of BMP signaling over time. To examine this, we analyzed the number of Olig2$^+$ cells in dorsal explants that were isolated at E10.5 or E12.5 and exposed to BMP7 or the BMP antagonists Noggin and Chordin (Piccolo et al., 1996; Zimmerman et al., 1996). In dorsal explants isolated at E10.5, the generation of Olig2$^+$ cells was completely blocked when cells were exposed to 1 ng/ml of BMP7 (Figure 5G; data not shown). Interestingly, the exposure of E10.5 dorsal explants to Noggin/Chordin resulted in a 3- to 4-fold increase in the number of Olig2$^+$ cells as compared to controls (Figures 5A, 5B, and 5E). This increase did not reflect a ventralization of progenitor identity, as indicated by the in vitro detection of Olig2$^+$/Pax7$^+$ cells (Figure 5F) and a lack of any detectable induction of Nkx2.2 or Nkx6.1 expression (data not shown). Instead, the increased number of Olig2$^+$ cells in E10.5 explants exposed to Noggin/Chordin correlated with a more rapid induction of these cells as compared to controls (Figures 5H–5L). BMP7 blocked Olig2 expression also in dorsal explants isolated 2 days later at E12.5 (data not shown). In contrast to explants isolated at E10.5, however, exposure of E12.5 explants to Noggin and Chordin did not result in an increased generation of Olig2$^+$ cells (Figures 5C–5E). While these data show that dorsal progenitors are still responsive to BMPs at E12.5, the failure of BMP antagonists to promote the generation of Olig2$^+$ cells at later stages is consistent with the idea that concentration of BMPs in the dorsal neural tube decreases over time.

Oligodendrocytes Derive from Different Ventral Progenitor Domains in the Spinal Cord and Hindbrain

While Nkx6 proteins are required for Olig2 expression in the ventral spinal cord, the expression of Olig2 persists in the ventral hindbrain of *Nkx6* mutants and is even ectopically activated at anterior hindbrain levels (Pattyn et al., 2003b). The differential regulation of Olig2 in the spinal cord and hindbrain raised the possibility that the generation of ventral oligodendrocytes is regulated differently at these axial levels. In support for this, and in contrast to the spinal cord, we found an extensive ventral ectopic induction of *Olig1*, Olig2, *Sox 10*, and PGDFRα in the anterior hindbrain of *Nkx6* mutants at E12.5 as compared to controls (Figures 6H, 6J, 6L, and 6N and data not shown). These data provide direct evidence that Nkx6 proteins suppress oligodendrocyte generation in ventral positions of the anterior hindbrain. Dorsally derived OLPs in the hindbrain, however, appeared to be unaffected by the loss of Nkx6 function (Figures 6G and 6H).

How then can Nkx6 proteins mediate opposing effects on the generation of oligodendrocytes at different axial levels of the CNS? Most HD transcription factors that are involved in ventral neural patterning, including Nkx6.1 and Nkx6.2, function directly as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data suggest that the promotion of Olig2 expression by Nkx6 proteins in the spinal cord is indirect and possibly involves an Nkx6-mediated exclusion of a repressor of Olig genes in pMN progenitors. In the spinal cord, the Olig1/2$^+$ pMN domain is located immediately dorsal to p3 progenitors, which express Nkx2.2 and produce *Sim1*-expressing V3 neurons (Briscoe et al., 1999). Nkx2.2 is sufficient and required for the generation of V3 neurons in the spinal cord and is an established repressor of Olig2 expression at this axial level (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Unexpectedly, we found that the domain of Nkx2.2 and *Sim1* expression had expanded dorsally and encroached into the presumptive pMN domain in the spinal cord of *Nkx6* mutants at E11.5 (Figures 6A–6D). These data show a genetic requirement for Nkx6 proteins to suppress Nkx2.2 expression in the pMN domain. Further, they reveal a strong correlation between the loss of Olig2 expression, and subsequent oligodendrogenesis, and the dorsal expansion of Nkx2.2 into the pMN domain of *Nkx6* mutant spinal cord.

The observation that loss of Nkx6 proteins in the anterior hindbrain instead results in an ectopic induction of OLP markers, led us to examine the generation of OLPS at this level in more detail. In the anterior hindbrain, ventral expression of Olig2 could first be detected at E12.5 (Figures 6E, 6I, and 6K). Most, if not all, Olig2$^+$

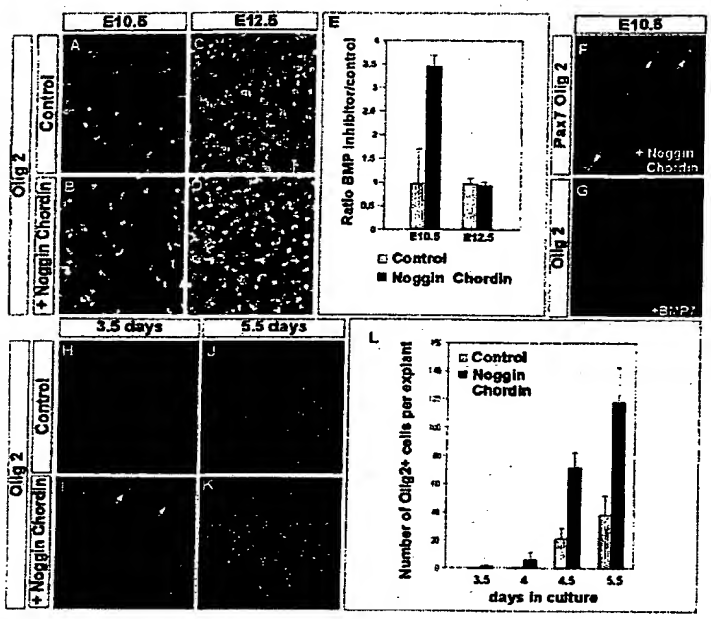

Figure 5. Decreased BMP Signaling Promotes the Generation of Dorsal Olig2$^+$ Cells in Vitro

(A–D) Olig2$^+$ cells in dorsal SC explants from E10.5 (A and B) and E12.5 (C and D) embryos cultured until corresponding day E18.5 in the absence (A and C) or presence (B and D) of BMP inhibitors Noggin and Chordin.

(E) Exposure of E10.5 explants to Noggin/Chordin resulted in a 3.5-fold increase in Olig2$^+$ cells as compared to controls. A similar number of Olig2$^+$ cells was observed in E12.5 explants cultured with or without Noggin/Chordin. Graph shows ratio of Olig2$^+$ cells in Noggin/Chordin-treated explants versus controls. Eight explants analyzed per time point; mean ± SD.

(F) Explants retain their dorsal identity in the presence of Noggin/Chordin as indicated by Pax7 expression and the detection of Pax7$^+$/Olig2$^+$ cells (arrows in [F]).

(G) BMP7 suppresses Olig2 expression in E10.5 dorsal SC explants cultured for 8 days.

(H–K) Olig2 expression in E10.5 dorsal explants cultured for various time points in the presence or absence of Noggin/Chordin.

(L) Quantification of Olig2$^+$ cells per explant. In explants exposed to Noggin/Chordin, Olig2$^+$ cells could first be detected after 3.5 days of culture, as compared to 4.5 days in controls. Explants treated with Noggin/Chordin show higher numbers of Olig2$^+$ cells compared to controls. Counts from six explants per time point; mean ± SD.

cells at this stage were located in lateral positions of the Nkx2.2$^+$ VZ, and many cells coexpressed Nkx2.2 and Olig2 (Figures 6E and 6I). Many Olig2$^+$ cells also expressed PGDFRα, indicating that they indeed are OLPs (Figure 6K). Thus, while Olig2$^+$ cells in the ventral spinal cord are generated dorsal to the Nkx2.2$^+$ progenitor domain, Olig1/2$^+$ cells in the anterior hindbrain appear to be derived from Nkx2.2-expressing progenitors. Like spinal cord levels, the domain of Nkx2.2 expression was expanded dorsally in the anterior hindbrain of *Nkx6* mutants at E12.5 (Figures 6F and 6J; data not shown). At this axial level, however, there was a striking correlation between the expanded expression of Nkx2.2 and the ectopic induction of oligodendrocyte differentiation (Figures 6J, 6L, and 6N). These data show that the differential regulation of oligodendrocytes by Nkx6 proteins is tightly linked to the expression of Nkx2.2 and to the distinct ventral origins of oligodendrocytes in the spinal cord and anterior hindbrain (Figures 6O and 6Q).

Discussion

Previous studies have established that oligodendrocytes in the spinal cord are generated from ventral pMN progenitors in the spinal cord. In addition to a ventral origin of these cells, we here provide evidence that oligodendrocytes are produced also by progenitors in the dorsal spinal cord and hindbrain. Our study further suggests that most ventrally generated oligodendrocytes in the hindbrain are produced from Nkx2.2$^+$ progenitors, rather than from more dorsally positioned pMN progenitors. Together, these data reveal multiple positional origins of oligodendrocyte specification in the spinal cord and hindbrain and provide evidence that the activation of Olig1/2 expression at different positions is regulated by distinct genetic programs.

A Dual Ventral and Dorsal Origin of Oligodendrocyte Generation in the Spinal Cord

sMNs and oligodendrocytes are generated sequentially from pMN progenitors in the ventral spinal cord, and the generation of these cells depends on the function of Olig1/2 (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). We provide several lines of evidence that, in addition to those in the pMN domain, dorsal progenitors in the spinal cord generate oligodendrocytes. First, at E15, approximately 2 days after the generation of OLPs from the pMN domain, we find that a subset of dorsal Olig2$^+$ cells are located within the VZ and coexpress the established dorsal progenitor markers Pax7 and Gsh1/2 (Figures 2B–2E). Second, oligodendrocytes are produced by isolated dorsal progenitors in culture, under in vitro conditions in which cells retain their dorsal progenitor identity. Olig2$^+$/Pax7$^+$ cells were generated in dorsal explants isolated as early as E10.5 and, together with our analysis of *Nkx6* mutants, these experiments argue against the formal possibility that a subset of pMN domain-derived OLPs would initiate Pax7 and Gsh1 expression after migrating into the dorsal neural tube. Third, while the pMN domain and ventral oligodendrocytes are missing in the spinal cord of *Nkx6* mutants, dorsal Olig2$^+$/Pax7$^+$/Gsh1/2$^-$ are generated on schedule and in numbers similar to those detected in controls at E15.

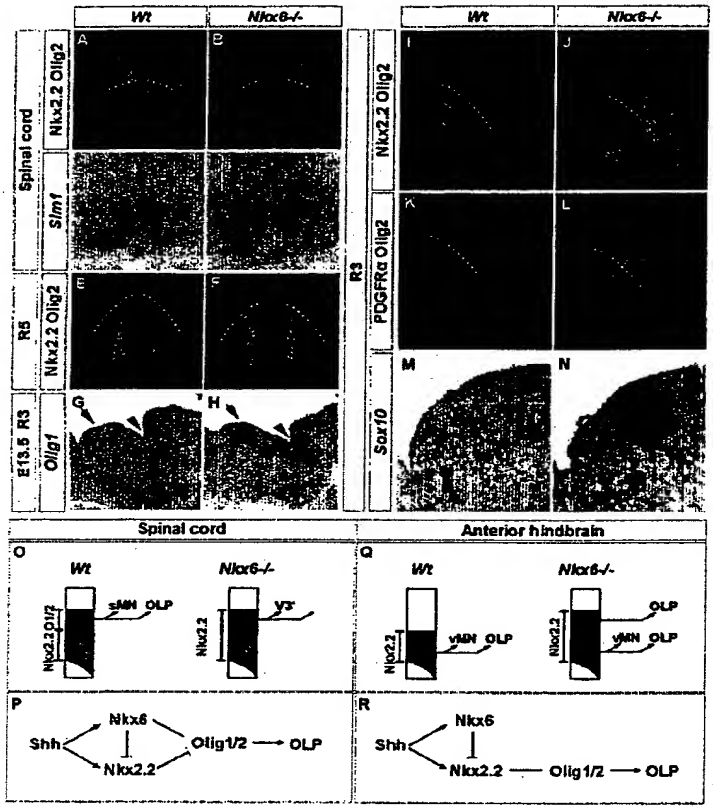

Figure 6. Different Ventral Progenitor Domains in Spinal Cord and Hindbrain Give Rise to Oligodendrocytes

(A–N) Transverse HB and SC sections of wt and *Nkx6* mutant embryos at E12.5–13.5. In the SC of wt mice, Olig2$^+$ cells are detected dorsal to the domain of Nkx2.2 expression at E12.5 (A). In *Nkx6* mutant mice, Olig2$^+$ cells are absent, and the expression of Nkx2.2 (B) and the V3 neuron marker *Sim1* (C and D) is dorsally expanded. Ventral Olig2$^+$ cells at the R5 and R3 levels of the E12.5 hindbrain are detected within, or in close proximity to, the Nkx2.2$^+$ domain (E and I). Note that several laterally positioned Nkx2.2$^+$ cells coexpress Olig2. In the *Nkx6* mutant HB, the domain of Nkx2.2 expression expands dorsally (F and J), and here the expansion is associated with an increased number and a dorsal expansion of Olig2$^+$ cells (F, J, K, and L). The generation of ectopic Olig2$^+$ cells in the ventral hindbrain of *Nkx6* mutants is accompanied by induction of OLP markers *Olig1* (G and H), PDGFRα (K and L), and *Sox10* (M and N). Dotted lines indicate dorsal boundary of wt Nkx2.2 expression. Arrow and arrowhead indicate ventral and dorsal Olig1+ domains, respectively (G and H). (O–R) Model of oligodendrocyte specification in the ventral spinal cord and hindbrain. In the ventral spinal cord and caudal-most hindbrain, somatic MNs and OLPs are sequentially generated from the Olig1/2$^+$ pMN domain located dorsal to Nkx2.2$^+$ progenitors (O). In the spinal cord, Shh induces expression of Nkx6 and Olig1/2 proteins in the pMN domain, while higher Shh concentrations induce Nkx2.2 expression in more ventral positions (P). Nkx6 proteins suppress Nkx2.2 expression in the pMN domain. The dorsal expansion of Nkx2.2 observed in *Nkx6* mutants may underlie the extinction of Olig1/2 expression in SC pMN progenitors and the ectopic generation of V3 neurons. Since Nkx6 proteins are coexpressed with Nkx2.2 in p3 progenitors, the repression of Nkx2.2 by Nkx6 is likely to require a pMN domain expressed cofactor. At anterior hindbrain levels, ventrally derived oligodendrocytes are generated from the Nkx2.2$^+$ pMNv domain that at earlier stages produce visceral MNs (Q and R). Nkx6 proteins control the dorsal limit of Nkx2.2 expression also in the hindbrain. Since Olig1/2-expressing OLPs derive from the Nkx2.2$^+$ progenitor at this level, the expansion of Nkx2.2 expression in the absence of Nkx6 function therefore results in a premature and ectopic initiation of Olig1/2 expression and oligodendrocyte differentiation (Q).

We cannot formally establish that dorsal Olig2$^+$ cells acquire properties of mature oligodendrocytes in vivo, since *Nkx6* mutants die at birth and, in wild-type embryos, differentiating dorsal Olig2$^+$ cells downregulate the expression of Pax7 and Gsh1/2 and intermingle with OLPs generated from the pMN domain. Nevertheless, our data show that cells in *Nkx6* mutants differentiate along the oligodendrocyte lineage in vivo and further that mature oligodendrocytes are produced in vitro in both cultured *Nkx6* mutant tissue and wild-type dorsal spinal cord explants. Taken together, these data strongly suggest that oligodendrocytes in the spinal cord are generated from both ventral and dorsal progenitor cells. Our study also suggests that oligodendrocytes are generated from dorsal progenitors in the hindbrain, and other studies have indicated a dual origin of oligodendrocytes in the forebrain (Gorski et al., 2002; Levison and Goldman, 1993). It is possible, therefore, that the specification of oligodendrocytes from dual, or multiple, DV positions is a general characteristic of the developing CNS.

The relative contribution of the dorsal lineage of OLPs is unclear, but comparisons of the total number of Olig1/2-expressing cells in control and *Nkx6* mutant embryos imply that dorsally generated cells represent a minor fraction of the total number of OLPs at prenatal stages of development (20%–30%; data not shown). In wild-type conditions, however, this number could be lower, since dorsally specified OLPs in *Nkx6* mutants could be propagated more efficiently due to the lack of ventrally derived OLPs that are likely to compete for essential growth factors such as PDGF (Calver et al., 1998). Also, while we observe similar numbers of Olig2$^+$ cells expressing dorsal progenitor markers in *Nkx6* mutant and controls at E15, the accompanying paper by Cai et al. (2005) in this issue of *Neuron* reports an approximately 3-fold increase in the number of Olig2$^+$/Pax7$^+$ cells in *Nkx6* mutants. While the reason for this difference between our studies remains unclear, it raises the possibility that the ventral loss of Nkx6 proteins and/or ventral oligodendrocytes may have a certain influence on the specification of OLPs from dorsal progenitor cells.

How then is the specification of dorsally derived oligo-

dendrocytes regulated? Olig1/2 proteins are required for the generation of all oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002), and a key step, therefore, must be to regulate the initiation of Olig1/2 expression in dorsal progenitor cells. Local BMP signaling from the roof plate has an essential early role in establishing dorsal neural tube identity and the patterned generation of dorsal neuronal subtypes (Helms and Johnson, 2003; Lee et al., 2000; Liem et al., 1997). Scattered Olig2$^+$/Pax7$^+$Gsh1/2$^+$ cells can first be detected in lateral positions of the VZ at around E15 (Figures 2B–2E, 4E, and 4G). We find that BMP7 suppresses oligodendrocyte differentiation in dorsal neural tube explants in vitro, while BMP antagonists enhance generation of dorsal Olig2$^+$ cells in dorsal explants isolated at early developmental stages. Since the spinal cord has grown considerably in size by E15, these data are consistent with a model in which the timing of Olig1/2 induction in dorsal progenitors involves a progressive evasion of BMP signaling due to a limited range of action of BMP signals secreted by the roof plate.

The induction of Olig1/2 expression in the pMN domain requires Shh signaling, raising the possibility that Shh also mediates the induction of Olig1/2 expression in the dorsal neural tube. This does not appear to be the case, since data by Cai and coworkers show that dorsal oligodendrocytes are generated in the absence of Shh signal transduction in vivo (Cai et al., 2005). FGF has been shown to promote Olig gene expression and oligodendrocyte differentiation in vitro (Chandran et al., 2003; Kessaris et al., 2004), but such experiments are difficult to interpret, since the induction of oligodendrocytes in response to FGFs in vitro has also been associated with an erroneous ventralization of progenitor cell identity (Gabay et al., 2003). To overcome this issue, we exposed isolated dorsal Pax7$^+$ progenitors to an inhibitor of FGF receptor signaling, SU 5402 (Mohammadi et al., 1997), and under these conditions we observed a complete block of Olig1/2 induction and oligodendrocyte differentiation (data not shown). While additional in vivo experiments are necessary to determine the precise role for BMP and FGF signaling in this process, these data indicate that a combination of FGF signaling and a progressive decrease in BMP activity over time may underlie the late phase of oligodendrocyte specification in the dorsal half of the spinal cord.

What is then the functional rationale of producing oligodendrocytes at several DV positions? One possibility is that a single origin of oligodendrocyte specification is not sufficient to produce the number of oligodendrocytes necessary to effectively insulate all axons at a given axial level. An alternative possibility is that the production of dorsal and ventral oligodendrocytes is necessary due to the establishment of physical or molecular barriers that hamper the migration of OLPs along the DV axis. Both these alternatives, however, appear to be unlikely since OLPs, once specified, are effectively propagated in a PDGF-dependent fashion outside of the VZ (Calver et al., 1998), and both ventral and dorsal OLPs appear to be capable of freely migrating along the DV axis of the spinal cord (Rowitch, 2004) (this study). The lack of Nkx2.2-expressing OLPs in *Nkx6* mutants implies that ventrally and dorsally derived OLPs express distinct molecular properties, at least at prenatal stages of development. An intriguing possibility, therefore, is that oligodendrocytes that are generated from distinct progenitor populations acquire distinct functional properties. Analyses of Olig1/2 function directly support that patterning along the DV axis controls the spatial specification of distinct glial cells, since the loss of oligodendrocytes in the pMN domain in *Olig1/2* mutants is associated with a concomitant gain of astrocytes (Zhou and Anderson, 2002). Also, in addition to the population of myelinating oligodendrocytes, certain oligodendrocytes have been shown to establish synapses with GABAergic interneurons in the hippocampus (Lin and Bergles, 2004). Clonal analyses in the postnatal forebrain have further revealed the presence of at least two types of OLPs, one that rapidly differentiates into myelinating oligodendrocytes and another that remains undifferentiated over extensive periods of time (Zerlin et al., 2004). While the mechanism(s) that underlies these functional, or behavioral, differences among oligodendrocytes remains to be determined, it is feasible that such differences will be related to the positional specification of oligodendrocytes along the DV axis of the neural tube.

Opposing Requirements for Nkx6 Proteins for Oligodendrocyte Specification in the Ventral Spinal Cord and Hindbrain

In addition to the identification of dorsally derived Olig2$^+$ OLPs in the spinal cord and hindbrain, our study reveals a striking difference between the specification of oligodendrocytes in the ventral spinal cord and hindbrain. The activity of Nkx6 proteins is required for the generation of oligodendrocytes from the ventral progenitors in the spinal cord, while the same proteins instead suppress oligodendrocyte production at anterior hindbrain levels (Figures 6O and 6Q). Our data indicate that this differential regulation reflects that oligodendrocytes derive from distinct ventral progenitor domains at these different axial levels. In the spinal cord, the pMN domain is located immediately dorsal to the Nkx2.2$^+$ p3 progenitor domain (Briscoe et al., 1999), and Nkx2.2 is an established repressor of Olig2 expression at this level (Novitch et al., 2001). A common phenotype in the spinal cord and hindbrain of *Nkx6* mutants is that the domain of Nkx2.2 expression expands dorsally, revealing a genetic requirement for Nkx6 proteins to control the dorsal limit of Nkx2.2 expression. It is conceivable, therefore, that a primary role of Nkx6 proteins in oligodendrogenesis in the ventral spinal cord is to indirectly ensure the maintained expression of Olig1/2 through the suppression of Nkx2.2.

Our analysis indicates that, in contrast to the spinal cord, the ventral oligodendrocytes in the anterior hindbrain are generated from the Nkx2.2$^+$ pMNv domain (Figures 6I and 6K) that at preceding stages has produced visceral MNs and serotonergic projection neurons (Ericson et al., 1997; Pattyn et al., 2003a, 2003b). As a consequence, the dorsal expansion of Nkx2.2 expression in the hindbrain of *Nkx6* mutants results in an increased production of OLPs rather than a loss of these cells. It remains unclear how Nkx2.2 can promote Olig1/2 expression in the hindbrain and why Nkx2.2 and Olig1/2 are coexpressed in hindbrain progenitors but not in the mouse spinal cord. Nevertheless, the expression of

Nkx2.2 at anterior hindbrain levels shows a mutually exclusive relationship with the expression of Irx3 (Pattyn et al., 2003b), a HD protein also known to repress Olig2 expression in the spinal cord (Novitch et al., 2001). Nkx2.2 could therefore promote Olig1/2 expression, at least in part, through the exclusion of Irx3 expression in the ventral-most part of the hindbrain.

Taken together, these data establish that oligodendrocytes in the ventral spinal cord and hindbrain are generated from distinct ventral progenitor domains. Although the specification of ventral oligodendrocytes at different axial levels shows a similarity with respect to their dependence on Shh signaling (Alberta et al., 2001; Lu et al., 2002) and requirement for Olig1/2, our analysis of *Nkx6* mutant mice reveals crucial differences in the intrinsic programs that control Olig1/2 expression in the ventral spinal cord and hindbrain (Figures 6P and 6R). Considering that the loss of Nkx6 function has no significant influence on oligodendrocytes specified in dorsal positions of the spinal cord and hindbrain, it is apparent that also the upstream control of Olig1/2 expression in dorsal progenitors must be differently regulated as compared to their ventral counterparts.

Experimental Procedures

Mouse Mutants

The generation and genotyping of Nkx6.1- and Nkx6.2-deficient mice have previously been reported (Sander et al., 2000; Vallstedt et al., 2001).

Neural Tube Explant Cultures

Rhombomeres 4–6 from hindbrains of E10.5 mouse embryos (CB57) were divided into ventral, intermediate, and dorsal portions. Explants were embedded in collagen (Cohesion Technologies) and cultured as previously described (Sussman et al., 2000), with the exception that 1% FBS was replaced with 1% KCR (Invitrogen). Spinal cords from E10.5 and E12.5 mouse embryos (CB57) were divided into ventral and dorsal portions and cultured under same conditions. For assessments of FGF effects, 20 ng/ml FGF2 (Invitrogen) was added to media. Functional blocking of FGF signaling was performed by adding 25 μM S5402 (Calbiochem) to the media. For assessments of BMP effects, 1 ng/ml BMP7 (R&D Systems) was added; blocking of BMP signaling was performed by adding 1 μg/ml Chordin and 1 μg/ml Noggin (R&D Systems).

Immunohistochemistry and In Situ Hybridization Histochemistry

Immunohistochemical localization of proteins was performed as described (Briscoe et al., 2000). Antibodies used were as follows: rabbit (r), mouse (m), and guinea pig (gp) Olig2 (Novitch et al., 2001), rat PDGFRα (PharMingen), m O4, rat MBP, r NG2 (Chemicon), m Pax7, m HB9, m Shh (DSHB), r Gsh1/2 (kind gift from Martin Goulding), m NeuN (Chemicon), r Nkx6.1, r Nkx2.2 (Briscoe et al., 2000). In situ hybridization histochemistry on sections or as whole mounts was performed (Schaeren-Wiemers and Gerfin-Moser, 1993) using mouse *Olig1*, *Olig2*, *Sox10*, *Dbx2*, *Nkx2.2*, *Pax3*, *Gsh1*, and *PDGFRα* probes.

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Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling

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Summary

In the developing spinal cord, early progenitor cells of the oligodendrocyte lineage are induced in the motor neuron progenitor (pMN) domain of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog* (*Shh*). The ventral generation of oligodendrocytes requires *Nkx6*-regulated expression of the bHLH gene *Olig2* in this domain. In the absence of *Nkx6* genes or *Shh* signaling, the initial expression of *Olig2* in the pMN domain is completely abolished. In this study, we provide the in vivo evidence for a late phase of *Olig* gene expression independent of *Nkx6* and *Shh* gene activities and reveal a brief second wave of oligodendrogenesis in the dorsal spinal cord. In addition, we provide genetic evidence that oligodendrogenesis can occur in the absence of hedgehog receptor *Smoothened*, which is essential for all hedgehog signaling.

Introduction

The spinal cord has served as an excellent model for studying the origin and molecular specification of oligodendrocytes in the developing central nervous system (CNS). Although oligodendrocytes are widely distributed in the adult spinal cord, recent findings have indicated that early progenitors of the oligodendrocyte lineage are induced from specific loci of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog* (*Shh*) (for reviews, see Richardson et al., 2000; Spassky et al., 2000; Miller, 2002). Under the influence of *Shh* morphogen, a number of transcription factors are selectively repressed (class I) or induced (class II) in the ventral neural progenitors (Briscoe et al., 2000), with each transcription factor having a different threshold response to the graded *Shh* signaling. As a result, these progenitor transcription factors display a nested pattern of expression along the dorsal-ventral axis. Based on their differential expression in the ventral spinal cord, the ventral neuroepithelium can be divided into five distinct domains (p0–p3 and motor neuron progenitor [pMN]), with each domain expressing a unique combination of progenitor genes and producing a specific neuronal cell type followed by either astrogliogenesis or oligodendrogenesis (Jessell, 2000; Zhou and Anderson, 2002). The pMN domain, which expresses *Nkx6* homeodomain transcription factors (Qiu et al., 1998; Briscoe et al., 2000; Vallstedt et al., 2001) and *Olig* bHLH transcription factors (Mizuguchi et al., 2001; Novitch et al., 2001), first produces motor neurons followed by oligodendrocyte precursor cells (OPCs) (Richardson et al., 1997; Sun et al., 1998; Fu et al., 2002) that subsequently migrate throughout the spinal cord before differentiating into myelinating oligodendrocytes. The sequential generation of motor neurons and OPCs from the pMN domain requires the expression of *Olig1* and *Olig2* transcription factors in this domain, and disruption of the *Olig* genes leads to the loss of both motor neurons and oligodendrocytes in the spinal cord (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Based on these and other observations, it is believed that, in the spinal cord, early OPCs originate from the pMN domain, and oligodendrocyte development is coupled to motor neuron development (Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002).

The possible contribution of dorsal neuroepithelium to oligodendrocyte development in the spinal cord has been under intensive investigation and considerable debate. In the developing chick embryos, some early transplantation studies suggested that oligodendrocytes were generated from both dorsal and ventral spinal cord (Cameron-Curry and Le Douarin, 1995). However, similar chick-quail grafting experiments argued that dorsal spinal neuroepithelial cells only produced astrocytes but not oligodendrocytes (Pringle et al., 1998). Recent studies in rodents suggested that glial-restricted progenitor (GRP) cells, which can give rise in vitro to OPCs and astrocytes, could be derived from both dorsal and ventral spinal cords (Rao et al., 1998; Gregori et al., 2002). Moreover, in vitro culture of dorsal mouse spinal cord explants, like that of its ventral counterpart, can also produce OPCs, although with a significant delay. In the mean time, the intermediate region located between the dorsal and ventral explants failed to generate OPCs in culture (Sussman et al., 2000), arguing against the possibility of dorsal invasion of OPCs from the ventral region. Together, these experiments indicated that the dorsal spinal neuroepithelial cells in mammals have an intrinsic and independent potential to produce oligodendrocytes under appropriate conditions. However, it is not known whether this potential is realized during the in vivo development of mouse spinal cord, as it has been argued that, in culture, neural progenitor cells may lose their positional cues and behave differently from in vivo in response to exogenous factors (Gabay et al., 2003; Stiles, 2003). For instance, bFGF can ventralize dorsal neural progenitor cells in vitro, resulting in an arbitrary induction *Olig2* expression and oligodendrocyte differentiation (Gabay et al., 2003; Chandran et al., 2003; Kessaris et al., 2004).

To investigate whether OPCs can be derived from the dorsal spinal cord in vivo, we examined oligodendrocyte development in *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{-/-}$ and *Shh*$^{-/-}$ mutant

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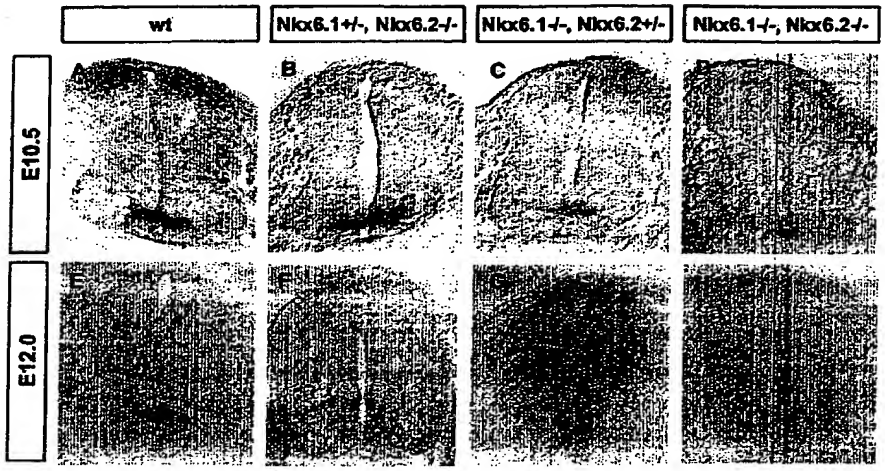

Figure 1. Early Expression of *Olig2* in Various *Nkx6* Mutant Spinal Cords

Transverse spinal cord sections from E10.5 (A–D) and E12.0 (E–H) embryos of various *Nkx* genotypes were subjected to in situ hybridization with *Olig2* riboprobe. The *Olig2* expression in the pMN domain was regulated by the redundant activities of *Nkx6.1* and *Nkx6.2* and was completely suppressed in $Nkx6^{-/-}$ double mutants.

spinal cords, in which the early ventral oligodendrogenesis from the pMN domain is abolished, so that the potential dorsal oligodendrogenesis could be unmasked. Our studies on oligodendrogenesis in $Nkx6^{-/-}$ double mutants and $Shh^{-/-}$ mutants uncovered a transient production of OPCs in the dorsal spinal cord. The dorsal generation of OPCs was also observed in wild-type spinal cords and was confirmed by in vitro culture of dorsal spinal cord explants. Together, these observations suggest an *Nkx*- and *Shh*-independent mechanism for *Olig* gene expression in the dorsal spinal cord after neurogenesis and provide evidence for a late phase of oligodendrogenesis independent of motor neuron development in the dorsal spinal cord.

Results

Nkx6 Dosage-Dependent *Olig* Gene Expression in the Ventral Ventricular Zone during Neurogenesis

Previous studies have demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification, with *Nkx6.1* having a larger effect than *Nkx6.2* (Vallstedt et al., 2001). To examine the effects of different levels of *Nkx6* gene activity on oligodendrocyte development in embryonic spinal cord, we first examined the early expression of *Olig2*, the principal *Olig* gene responsible for motor neuron and oligodendrocyte development (Lu et al., 2002; Takebayashi et al., 2002), in the ventral spinal cords of various *Nkx6* mutants prior to oligodendrogenesis stages. Consistent with the previous findings (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000), at E10.5 and E12.0, *Olig 2* was exclusively expressed in the pMN domain of the wild-type spinal cord (Figures 1A and 1E). In heterozygous embryos ($Nkx6.1^{+/-}$, $Nkx6.2^{+/-}$, or $Nkx6.1^{+/-}$ $Nkx6.2^{+/-}$) and $Nkx6.2^{-/-}$ homozygous embryos, *Olig2* expression was not significantly affected (data not shown). In $Nkx6.1^{+/-}$ $Nkx6.2^{-/-}$ embryos, expression of *Olig2* in the ventral ventricular zone was slightly decreased (Figures 1B and 1F). However, *Olig2* expression was markedly reduced in $Nkx6.1^{-/-}$ (Liu et al., 2003) or $Nkx6.1^{-/-}$ $Nkx6.2^{+/-}$ embryos (Figures 1C and 1G) and completely eliminated in $Nkx6.1^{-/-}$ $Nkx6.2^{-/-}$ (referred to as $Nkx6^{-/-}$ hereafter) compound mutants (Figures 1D and 1H). Collectively, these results indicated a dosage-dependent regulation of *Olig2* expression in the ventral spinal cord by *Nkx6* transcription factors, and its expression is largely dependent on *Nkx6.1* activity but to a lesser extent on *Nkx6.2* activity.

Delayed and Dorsal Expression of *Olig* Genes in $Nkx6^{-/-}$ Spinal Cords during Gliogenesis

To investigate whether the lack of *Olig* expression in the pMN domain leads to a complete inhibition of oligodendrogenesis in the spinal cord, we examined OPC generation and differentiation at progressively later stages of embryonic development in $Nkx6^{-/-}$ double mutants. At E13.5, many *Olig1*+ and *Olig2*+ OPCs had already migrated out of the ventral ventricular zone in wild-type spinal cords (Figures 2A and 2C). As expected, no migratory *Olig*+ cells were observed outside the ventricular zone in $Nkx6^{-/-}$ mutants. Surprisingly, a small number of *Olig1*+ and *Olig2*+ cells were detected in the mutants in both dorsal and ventral ventricular zone (Figures 2B and 2D). The ventral expression of *Olig* genes in $Nkx6^{-/-}$ mutants occurred at approximately the same position as the pMN domain. These data suggested a *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord during oligodendrogenesis stages. The dorsal expression of *Olig* genes in the mutants became more apparent at E14.5, when a small number of *Olig1*+ and *Olig2*+ OPCs started to

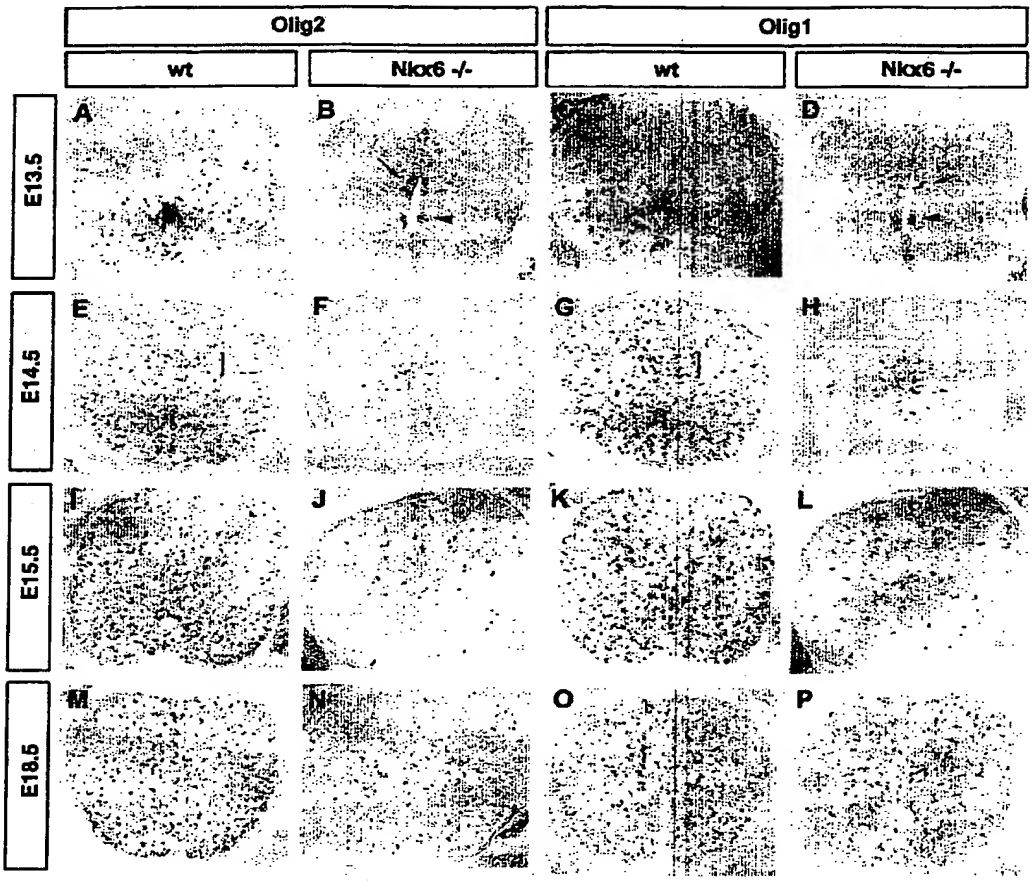

Figure 2. Late and Dorsal Expression of *Olig2* Gene in *Nkx6*$^{-/-}$ Spinal Cords at the Thoracic Level

Transverse sections from E13.5 (A–D), E14.5 (E–H), E15.5 (I–L), and E18.5 (M–P) spinal cords of wild-type and *Nkx6*$^{-/-}$ embryos were subjected to in situ hybridization with riboprobes for *Olig2* and *Olig1*. At E13.5, *Olig1* and *Olig2* expression was upregulated in *Nkx6*$^{-/-}$ double mutants in both dorsal (indicated by arrows) and ventral (indicated by the arrowheads) positions. At E14.5 and later stages, *Olig1*+ and *Olig2*+ cells migrated into the surrounding regions in a dorsal to ventral gradient, in contrast to that seen in the wild-type spinal cords. The positions of dorsal-derived *Olig*+ cells in E14.5 wild-type spinal cord are outlined by a square bracket in (E) and (G).

migrate away from the dorsal ventricular zone (Figures 2F and 2H). Interestingly, very few migratory OPCs were produced from the *Olig*-expressing ventral ventricular zone of the mutant spinal cord. Thus, a vast majority of *Olig*+ OPC cells in *Nkx6*$^{-/-}$ mutants appeared to originate from the dorsal ventricular zone. At this stage, a distinct population of *Olig1*+ and *Olig2*+ cells were also closely associated with the dorsal ventricular or subventricular zone of the wild-type spinal cords (Figures 2E and 2G), and there was an apparent discontinuity between this group of *Olig*+ cells and the ventral-derived *Olig*+ cells (more apparent in Figures 5A, 5I, 8C, and 8E). Together, these observations suggest that a small number of *Olig*+ OPCs are produced from the dorsal neuroepithelial cells in both normal and *Nkx6*$^{-/-}$ spinal cords.

At E15.5, the number of *Olig1/2*+ cells in *Nkx6*$^{-/-}$ mutants was further increased, but most of them remained confined to the dorsal spinal cords (Figures 2J and 2L). In contrast, a higher percentage (55%) of *Olig1/2*+ OPCs were found in the ventral half of wild-type spinal cord (Figures 2I and 2K), and they were presumably derived from the ventral neuroepithelial cells. By E18.5, *Olig1/2*+ cells were distributed more or less evenly throughout the entire spinal cord in *Nkx6*$^{-/-}$ mutants (Figures 2N and 2P), suggesting that the dorsal-derived *Olig*+ OPCs in *Nkx6*$^{-/-}$ mutants migrated progressively from the dorsal to the ventral spinal cord. However, the number of *Olig*+ cells remained significantly smaller than that in wild-type embryos (Figures 2M and 2O; Figure 3Q).

Delayed Appearance of Other Oligodendrocyte Markers in *Nkx6*$^{-/-}$ Mutants

One critical issue for the dorsal-derived *Olig*+ cells is whether they are capable of differentiating further along the oligodendrocyte lineage. To address this question,

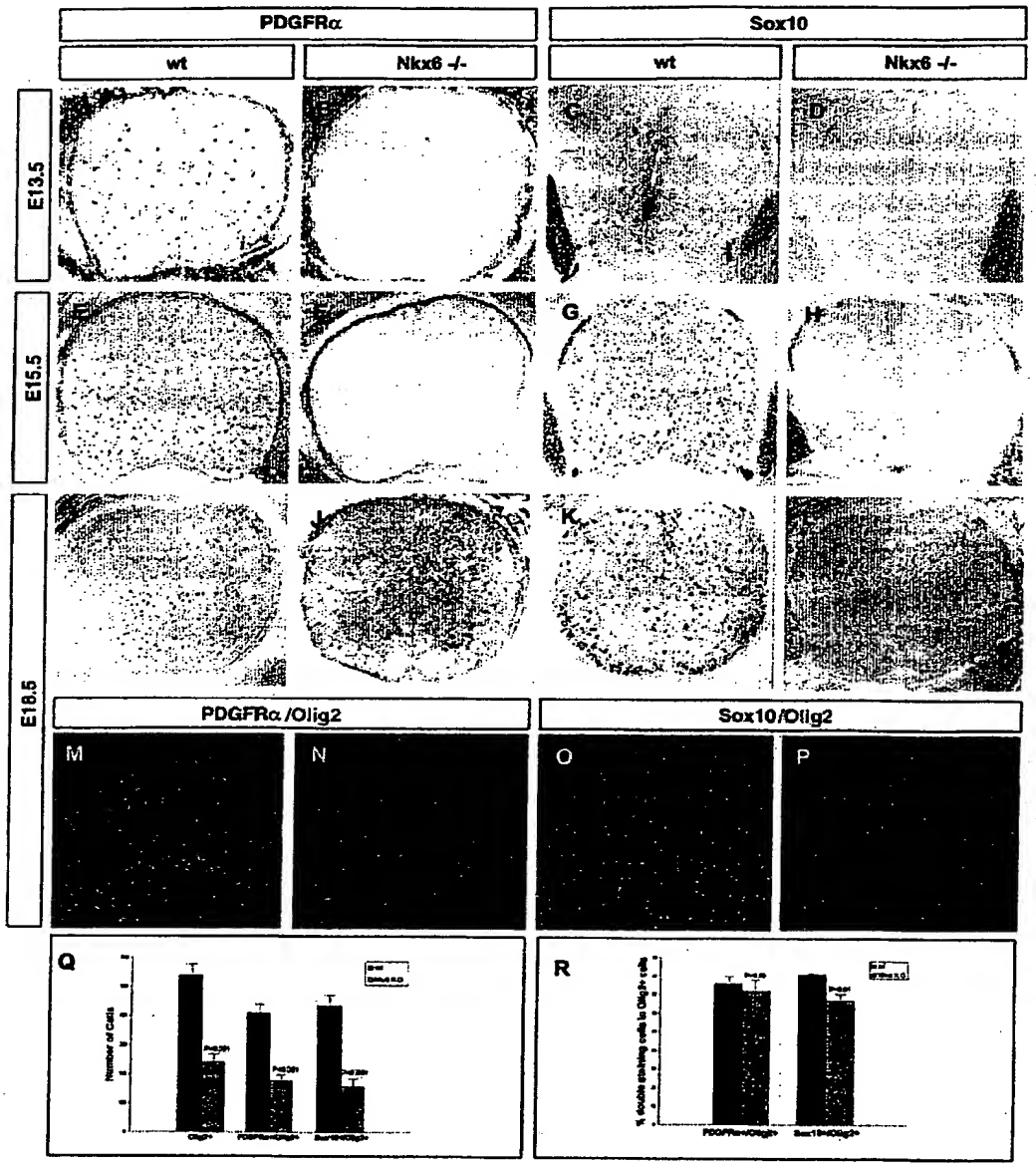

Figure 3. Delayed and Reduced Production of *PDGFRα*+ and *Sox10*+ OPCs in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–L) Spinal cord sections from E13.5 (A–D), E15.5 (E–H), and E18.5 (I–L) wild-type and mutant embryos were subjected to in situ hybridization with *PDGFRα* or *Sox10* riboprobes. A smaller number of *PDGFRα*+ and *Sox10*+ cells started to emerge in E18.5 *Nkx6*$^{-/-}$ spinal cords.

(M–P) Double immunostaining of E18.5 wild-type and mutant spinal cord with *Olig2* (green) and *PDGFRα* ([M] and [N], red) or *Sox10* ([O] and [P], red).

(Q) The number of Olig2+ single-positive OPCs and *Olig2*+/*PDGFRα*+ or *Olig2*+/*Sox10*+ double-positive cells per spinal cord section in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants (average of three sections).

(R) The percentage of *Olig2*+ cells that coexpress *PDGFRα* or *Sox10* in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants. Statistical analyses in (Q) and (R) were performed with Student's t test.

we examined the expression of several oligodendrocytic markers (e.g., *PDGFRα*, *Sox10*, and *MBP*) downstream of *Olig1/2* in *Nkx6*$^{-/-}$ spinal cords. In wild-type spinal cords, expression of *PDGFRα* and *Sox10* is restricted to oligodendrocyte lineage (Pringle et al., 1992; Stolt et al., 2002) and can be detected in the ventral spinal cord at E13.5, whereas their expression in *Nkx6*$^{-/-}$ mutants was not observed until E18.5 (Figures 3A–3L), indicating

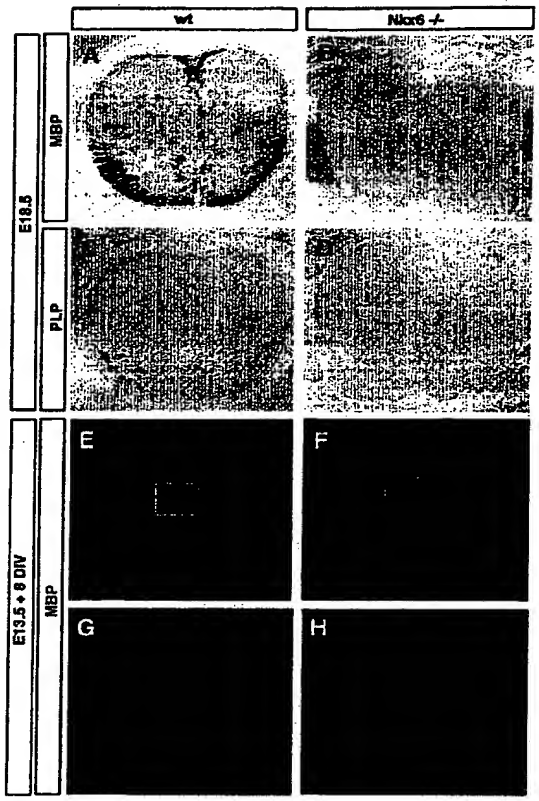

Figure 4. Disrupted Expression of *MBP* and *PLP* in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–D) Spinal cord sections were prepared from E18.5 wild-type (A and C) and mutant (B and D) animals and hybridized with *MBP* (A and B) and *PLP* (C and D) riboprobes.

(E–H) Spinal cord explants isolated from E13.5 wild-type and *Nkx6*$^{-/-}$ embryos were cultured on floating membranes for 8 days before they were subjected to anti-*MBP* whole-mount immunostaining. (G) and (H) are higher magnifications of (E) and (F), respectively, showing *MBP*+ myelinating axons.

a significant delay of OPC differentiation. Double immunostaining at this stage confirmed that a high percentage of *Olig2*+ cells in *Nkx6* mutants coexpressed *PDGFRα* and *Sox10* (Figures 3M–3P and 3R), although the total number of *Olig2*+/*PDGFRα*+ and *Olig2*+/*Sox10*+ cells per spinal cord section remained significantly smaller (Figure 3Q). Similarly, expression of the mature oligodendrocyte markers *MBP* and *PLP* in mutant spinal cords was also affected. In normal embryos, many *MBP*+/*PLP*+ oligodendrocytes were seen in the ventral spinal cord at E18.5 (Figures 4A and 4C). However, no *MBP*+/*PLP*+ cells were detected in *Nkx6*$^{-/-}$ mutants at this stage (Figures 4B and 4D). Together, these results suggest that the dorsal-derived *Olig*+ cells in *Nkx6*$^{-/-}$ spinal cords can progress along the oligodendrocyte lineage, but they develop and mature much more slowly than the early-born ventral OPCs.

To assess whether dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into mature oligodendrocytes in vitro, we isolated spinal cord explants from E13.5 wild-type and mutant embryos and cultured them on floating membranes. Following 8 days of culture, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 4E and 4F). Moreover, *MBP*+ fibers, indicators of myelinating axons, were observed in the axon-enriched medial (ventral) regions of both normal and mutant explants (Figures 4G and 4H). These results suggest that the dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into *MBP*+ mature oligodendrocytes and form myelin sheaths, at least in vitro.

Olig+ OPCs Are Briefly Produced from the Dorsal Neuroepithelial Cells and Transiently Coexpress Some Dorsal Neural Progenitor Markers

To verify the dorsal origin of a subset of *Olig*+ OPCs in both normal and *Nkx6*$^{-/-}$ spinal cords, sections from E14.5 embryos were subjected to double immunostaining with antibodies against *Olig2* and two dorsal neural progenitor markers, *Pax7* and *Mash1*. During neurogenesis and early gliogenesis, *Pax7* is expressed in the entire dorsal ventricular zone of the spinal cord (Goulding et al., 1993), whereas *Mash1* expression in the dorsal spinal cord is restricted to the dorsal interneuron progenitor domains dI3–dI5 (Gross et al., 2002; Muller et al., 2002; Caspary and Anderson, 2003). Double immunostaining revealed that a subpopulation of *Olig2*+ cells was closely associated with the *Pax7*+ and *Mash1*+ dorsal neuroepithelial cells in both genotypes (Figures 5A–5J). Moreover, a small number of migratory *Olig2*+ cells in the dorsal ventricular zone or immediately adjacent regions coexpressed *Pax7* and *Mash1* (arrows in Figures 5B–5D and 5F–5H; insets in Figures 5I and 5J). These colabeling data strongly suggested that some *Olig2*+ cells arose from the dI3–dI5 domains of dorsal neural progenitor cells in both normal and *Nkx6*$^{-/-}$ spinal cords and that the dorsal-derived *Olig2*+ cells retained the expression of dorsal markers *Pax7* and *Mash1* for a brief period of time. Although only about 8% of *Olig2*+ cells were also *Pax7*+ in E14.5 wild-type spinal cord, this may be an underestimate of the percentage of dorsal-derived OPC population due to the rapid downregulation of *Pax7* after they migrate away into the surrounding region. Intriguingly, the total number of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ cells in *Nkx6*$^{-/-}$ double mutants was significantly larger than that in normal embryos (Figure 5M). One plausible explanation is that the dorsal-derived *Olig2*+ cells in mutant spinal cords proliferated more rapidly, possibly due to the lack of competition from the ventral-derived OPCs for mitogens. Alternatively, expression of *Pax7* and *Mash1* in dorsal-derived OPCs may be downregulated more slowly in *Nkx6* mutants.

To confirm our mapping of the origin of dorsal OPCs, we also compared the expression of *Olig2* with that of two other neural progenitor genes, *Dbx1* and *Dbx2*. Previous studies have shown that *Dbx1* is expressed in the dorsal-ventral boundary of the embryonic spinal cord, whereas *Dbx2* is expressed in the dI6 domain of the dorsal spinal cord and the p0 and p1 domains of the ventral spinal cord (Briscoe et al., 2000; Caspary

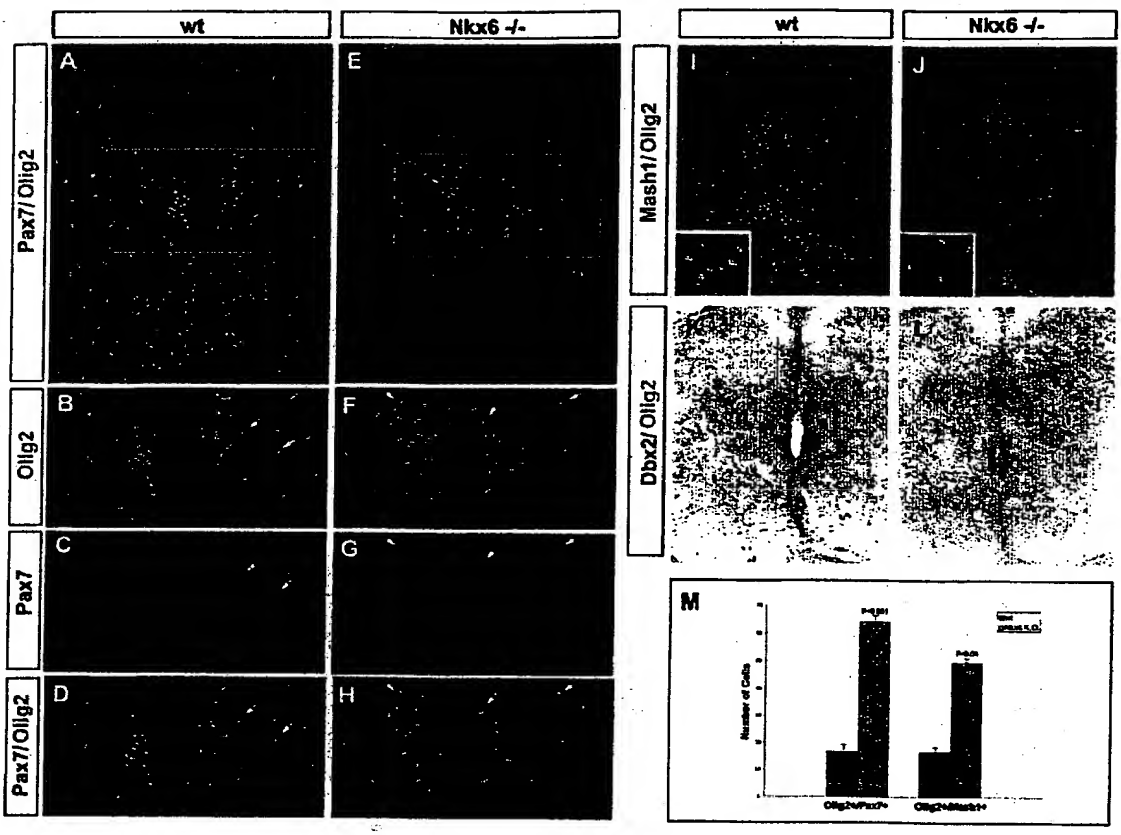

Figure 5. *Olig*+ Cells Originated from *Pax7*+ and *Mash1*+ but *Dbx*− Dorsal Interneuron Progenitor Domains

(A–H) Coexpression of *Pax7* and *Olig2* in the dorsal spinal cord. E14.5 spinal cord sections from wild-type (A–D) and *Nkx6*$^{-/-}$ (E–H) embryos were simultaneously immunostained with antibodies against *Olig2* (in green) and *Pax7* (in red). (B)–(D) and (F)–(H) are the higher magnifications of the boxed areas in (A) and (E), respectively. In both genotypes, a group of *Olig2*+ cells were produced from the *Pax7*+ dorsal ventricular zone, and some of the *Olig2*+ cells retained the expression of *Pax7* (represented by arrows in [B]–[D] and [F]–[H]).

(I–L) E14.5 wild-type and *Nkx6*$^{-/-}$ spinal cord sections were double immunostained with anti-*Olig2* and anti-*Mash1* antibodies (I and J), or subject to in situ hybridization with *Dbx2* riboprobe followed by anti-*Olig2* immunohistochemistry (K and L). The *Olig2*+/*Mash1*+ double-positive cells are represented in insets in (I) and (J). The dorsal-derived *Olig2*+ cells in (K) and (L) are outlined by a square bracket.

(M) Statistical analyses (Student's t test) of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ double-positive cells in wild-type and *Nkx6*$^{-/-}$ mutants per section.

and Anderson, 2003). Double labeling of E14.5 spinal cord sections demonstrated that the dorsal *Olig2*+ cells lay immediately dorsal to *Dbx2* expression (Figures 5K and 5L) but well above *Dbx1* expression (data not shown), indicating that the dorsal *Olig2*+ cells were derived from regions above the dI6 domain. This result is consistent with the idea that dorsal *Olig2*+ cells are primarily derived from the *Mash1*+ dI3–dI5 dorsal interneuron progenitor cells.

To further confirm that the dorsal spinal cord has an independent potential to generate OPCs, we dissected the dorsal and ventral halves of spinal cord from E11.5 mouse embryos and cultured them separately in collagen gel or on floating membranes in the absence of exogenous bFGF, which is known to induce *Olig2* expression in cultured dorsal neural progenitor cells (Gabay et al., 2003; Chandran et al., 2003). In E11.5 mouse spinal cord, OPCs were not produced from the pMN domain yet (Figures 6A and 6B), excluding the possibility of dorsal invasion of ventral OPCs. Following 3 days of in vitro culture (equivalent to E14.5), a small number of *Olig2*+ cells started to emerge from the dorsal explants and coexpressed Pax7 (Figure 6C). Expression of later OPC markers *PDGFRα* and NG2 in dorsal explants was seen after 4–6 days of culture, and that of mature markers GalC and *MBP* was seen after 6–8 days of culture in vitro (Figures 6E–6L). Together, these data indicated that the dorsal spinal cord explants have an intrinsic potential to produce OPCs, and the schedule of OPC generation and differentiation in dorsal explant culture

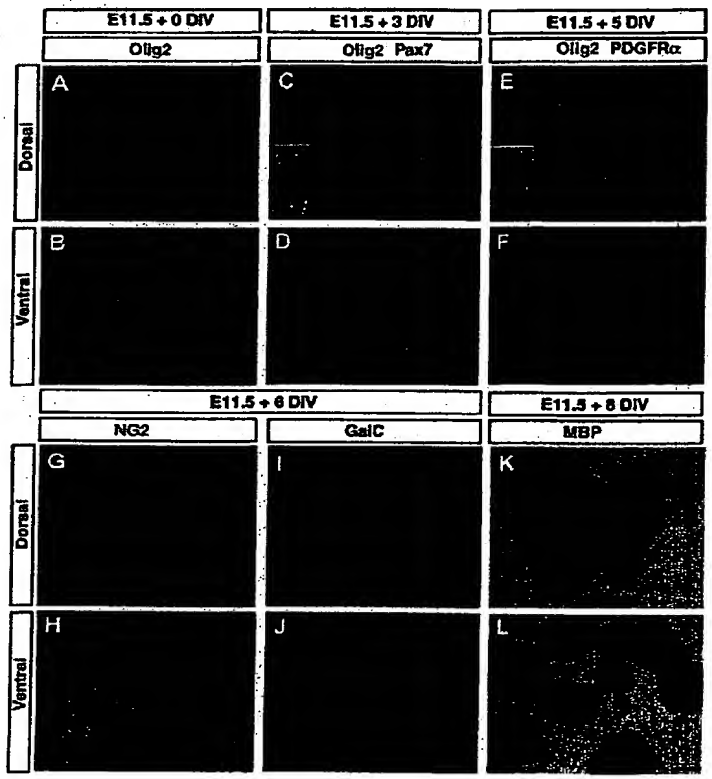

Figure 6. Generation of Oligodendrocytes from Dorsal Spinal Cord Explant Culture

Mouse E11.5 spinal cord tissues isolated from the thoracic level were bisected into dorsal and ventral halves and cultured separately in collagen gel (A–J) or on floating membrane (K and L) for various days in vitro (DIV) as indicated. Explants were then subject to immunofluorescent staining with antibodies or in situ hybridization with *MBP*.

is similar to that in vivo. In agreement with previous findings (Gabay et al., 2003; Chandran et al., 2003), addition of exogenous bFGF dramatically increased the number of *Olig2*+ OPCs in both dorsal and ventral explants (data not shown).

Generation of Dorsal OPCs in the Absence of *Shh* Signaling

The generation of OPCs in the dorsal spinal cord suggests a *Shh*-independent pathway for oligodendrogenesis, since cell fate specification in the dorsal spinal cord is primarily regulated by dorsal midline signals, notably BMPs (Dickinson et al., 1995; Liem et al., 1995). To test the possibility, we examined whether OPCs' production from dorsal explants can be blocked by anti-*Shh* antibody. In contrast to the previous finding that *Shh* was partially required for O4 expression in dorsal explants (Sussman et al., 2000), we found that anti-*Shh* antibody had no apparent effect on *Olig2* gene expression in dorsal explants, although it dramatically inhibited *Olig2* expression in the ventral explants (Figures 7A–7D). To provide genetic evidence that oligodendrogenesis can occur independent of hedgehog signaling, we differentiated ES (embryonic stem) cells deficient in pan-hedgehog signaling component *Smoothened* (*Smo*$^{-/-}$; Wijgerde et al., 2002) in the presence of retinoid acid and found that GalC+ oligodendrocytes were formed from both normal and *Smo*$^{-/-}$ mutant ES cells at a comparable efficiency (Figures 7E and 7F).

We next examined oligodendrocyte development in *Shh*$^{-/-}$ mutant spinal cord to confirm that *Shh* signaling is not responsible for dorsal oligodendrogenesis in vivo. In *Shh*$^{-/-}$ mutants, the spinal cord is dorsalized, and most of the ventral structures including the pMN domains are missing (Chiang et al., 1996; Pierani et al., 1999). Consistent with some earlier findings that *Shh* is required for ventral oligodendrogenesis (Lu et al., 2000; Alberta et al., 2001), no early *Olig1/2*+ OPCs were produced in *Shh* mutant spinal cords at or before E13.5 (Figures 8A and 8B; data not shown). However, at E14.5, a small number of *Olig1*+ and *Olig2*+ cells started to appear in the dorsal region of the mutant spinal cords (Figures 8D and 8F). By E18.5, a larger number of *Olig2*+ cells were observed throughout the mutant spinal cord (Figure 8H).

Similar to our data in *Nkx6*$^{-/-}$ mutants, oligodendrocyte lineage progression in *Shh* null spinal cord was also delayed. Expression of *PDGFR* and *Sox10* was not detected until E18.5 (Figures 8I–8L), and no *MBP* expression was observed at perinatal stages (Figures 8M and 8N). However, when spinal cord explants isolated from E18.5 mutant embryos were cultured in vitro for 2 additional days, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 8O and 8P), indicating

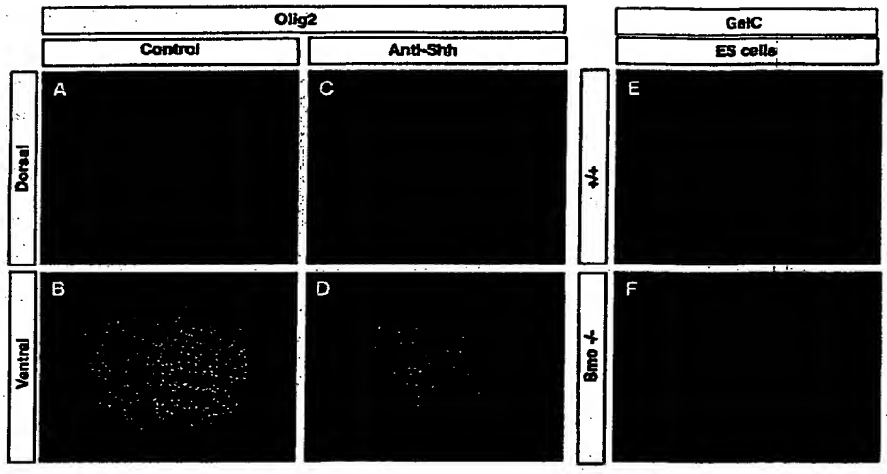

Figure 7. *Shh*-Independent Generation of Oligodendrocytes in Spinal Explants and ES Cells

(A–D) Inhibition of *Olig* gene expression by anti-*Shh* antibody in ventral explants but not in dorsal explants. Dorsal and ventral spinal cord explants from E11.5 wild-type embryos were cultured in collagen gel for 3 days in the absence (A and B) or presence (C and D) of anti-*Shh* antibody prior to immunostaining with anti-*Olig2*.

(E and F) Differentiation of wild-type (E) and *Smo*$^{-/-}$ (F) ES cells into GalC+ oligodendrocytes.

that the dorsal-derived OPCs in *Shh* mutants are able to differentiate into *MBP*+ mature oligodendrocytes as well.

Discussion

This study reveals an *Nkx6*- and *Shh*-independent mechanism for a late phase of *Olig* gene expression in the dorsal spinal cord after the onset of early oligodendrogenesis from the pMN domain (Figure 9). The late *Olig* gene expression is associated with a brief wave of oligodendrogenesis in the dorsal spinal cord. These findings provide evidence for multiple origins of OPC generation involving distinct inductive signals during spinal cord development.

Nkx6-Independent Mechanisms for *Olig* Gene Expression and Oligodendrogenesis in the Spinal Cord

Previous work had demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification in the spinal cord (Vallstedt et al., 2001). Consistent with this line of study, our findings indicate that *Nkx6.1* and *Nkx6.2* have redundant activities in regulating the early expression of *Olig2* in the pMN domain, with *Nkx6.1* exerting a larger effect than *Nkx6.2* (Figure 1). In the absence of both *Nkx6.1* and *Nkx6.2*, the initial expression of *Olig 2* in the pMN domain is completely abolished. In keeping with the idea that early progenitors of the oligodendrocyte lineage are derived from the *Olig2*+ pMN domain of the ventral spinal cord, the loss of *Olig2* expression in the pMN domain in *Nkx6*$^{-/-}$ mutants was associated with the failure of production of early OPC cells from the ventral spinal cord (Figure 2).

Despite the lack of early expression of *Olig2* in the pMN domain in *Nkx6*$^{-/-}$ mutants, a low level of *Olig1* and *Olig2* expression started to be detected in both the ventral and dorsal ventricular zone after the onset of oligodendrogenesis (Figure 2). In the dorsal spinal cord, *Olig* gene expression was detected in *Pax7+/Mash1+* dI3–dI5 dorsal neural progenitor domains starting at E13.5 (Figures 2 and 5). In the ventral spinal cord, a small number of ventral ventricular cells started to express *Olig1* and *Olig2* genes at approximately the same position as the pMN domain (Figures 2B and 2D). Together, these results indicate an *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord. The late phase of *Olig* gene expression in the dorsal spinal neuroepithelium was also observed in wild-type spinal cord, mostly at E14.5 (Figures 5 and 8) but occasionally at E13.5 (data not shown). Thus, the dorsal ventricular *Olig2* expression appeared to be slightly advanced or enhanced in *Nkx6* mutants. This enhancement might partially account for the increased population of *Olig2+/Pax7+* cells in the mutants. Interestingly, no *Olig* expression was observed in E13.5 *Shh* mutants (Figure 8), suggesting that the premature or enhanced dorsal ventricular *Olig* expression was not simply due to the loss of ventral patterning, but more specifically associated with the absence of *Nkx6* gene expression.

The dorsal *Olig* gene expression was associated with a transient production of OPCs starting at around E14.5, about 2 days later than the ventral oligodendrogenesis from the pMN domain (Figure 9B). In both wild-type and *Nkx6*$^{-/-}$ spinal cords, migratory *Olig1/2*+ OPCs were briefly produced from the dorsal neural progenitor cells. Several lines of evidence strongly suggest that these OPC cells are generated de novo from the dorsal ventricular cells, instead of having migrated up from the ventral cord. First, many dorsal *Olig2*+ cells coexpressed sev-

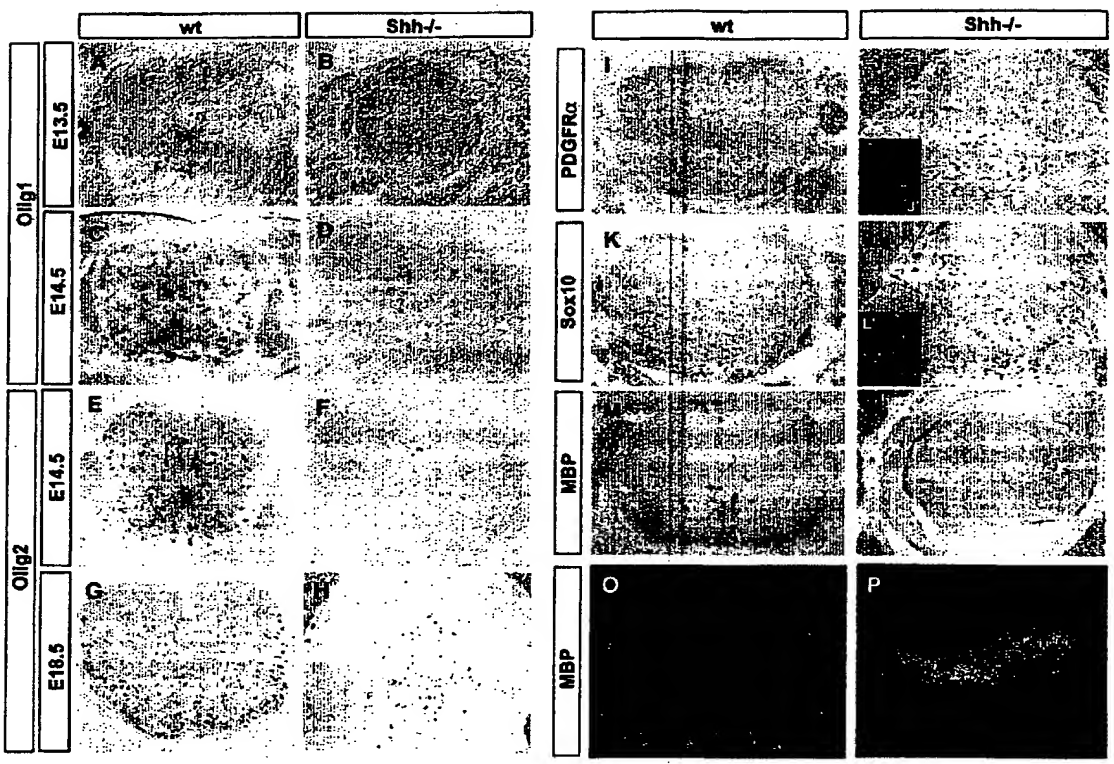

Figure 8. Oligodendrocyte Development in *Shh* Mutant Spinal Cord

(A–H) Dorsal generation of *Olig1/2*+ OPC cells in *Shh* mutant spinal cords. Spinal cord sections from E13.5 (A and B), E14.5 (C–F), and E18.5 (G and H) wild-type (A, C, E, and G) or *Shh*$^{-/-}$ (B, D, F, and H) embryos were subjected to in situ hybridization (ISH) with *Olig1* (A–D) and *Olig2* (E–H) riboprobes. At E13.5, *Olig*+ OPCs were generated from the ventral neuroepithelium in wild-type embryos but not in *Shh* mutants. At E14.5, a small group of *Olig1*+ and *Olig2*+ cells were associated with the dorsal neuroepithelium in both wild-type and *Shh* mutants. Dorsal *Olig*+ cells are outlined by a square bracket in (C) and (E).

(I–N) Distribution and differentiation of OPC cells in *Shh* mutant spinal cords. (I–N) Expression of *PDGFRα* (I and J), *Sox10* (K and L), and *MBP* (M and N) in E18.5 wild-type (I, K, and M) and *Shh* mutant (J, L, and N) spinal cords. *Olig2*+/*PDGFRα*+ and *Olig2*+/*Sox10*+ cells in mutants are represented in insets (J′) and (L′), respectively.

(O and P) Spinal cord tissues from E18.5 wild-type and *Shh* mutant embryos were isolated and cultured on polycarbonate membranes for 2 days in vitro and then subjected to whole-mount ISH with the *MBP* probe. A small number of *MBP*+ cells emerged in the mutant tissue.

eral dorsal neural progenitor genes such as *Pax7* and *Mash1* (Figure 5). Second, dorsal neural explants isolated from E11.5 spinal cord prior to ventral oligodendrogenesis can give rise to OPCs and oligodendrocytes on schedule as in vivo (Figure 6). Third, dorsal *Olig2* expression in *Nkx6* mutants was no later than its ventral expression. At E13.5 and E14.5, there was an apparent discontinuity of dorsal *Olig2*+ cells and ventral *Olig2*+ cells in the mutants (Figures 2B, 2D, 5E, and 5J), and the number of *Olig2*+ cells in E14.5 dorsal half far exceeded that of ventral *Olig2*+ cells, arguing against the dorsal migration of *Olig2*+ cells at least at these early stages. However, it is plausible that some ventral *Olig2*+ cells could migrate dorsally after E14.5 and contribute to the dorsal OPC population. Finally, OPCs can be produced from the dorsal region of *Shh* mutant spinal cord, which lacks the pMN domain (Pierani et al., 1999) and presumably the ventral oligodendrogenesis (Figure 8), although we can not absolutely exclude the possibility that a few *Olig*+ cells could also be generated from the remaining p0 and p1 domains in the most ventral region (Pierani et al., 1999). Together, these observations indicate that dorsal *Olig2*+ cells can be produced locally from the dorsal neuroepithelial cells in normal and *Nkx6*/*Shh* mutant spinal cords. Since the dorsal *Olig2*+/*Pax7*+/*Mash*+ OPCs were observed in the thoracic or even more caudal regions (data not shown), it is unlikely that they represent the longitudinally migrating cells from the rostral hindbrain.

Similar to the early ventral OPCs, the dorsal-derived *Olig*+ OPCs are capable of migration, proliferation, and differentiation along the oligodendrocytic lineage after they migrate out of the germinal zone. The delayed appearance of *PDGFRa*+ and *Sox10*+ OPCs in the *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutants indicated that the late-born dorsal OPCs develop and differentiate much later than the early-born ventral oligodendrocytes. Although no *MBP* and *PLP* expression was observed in both mutants at

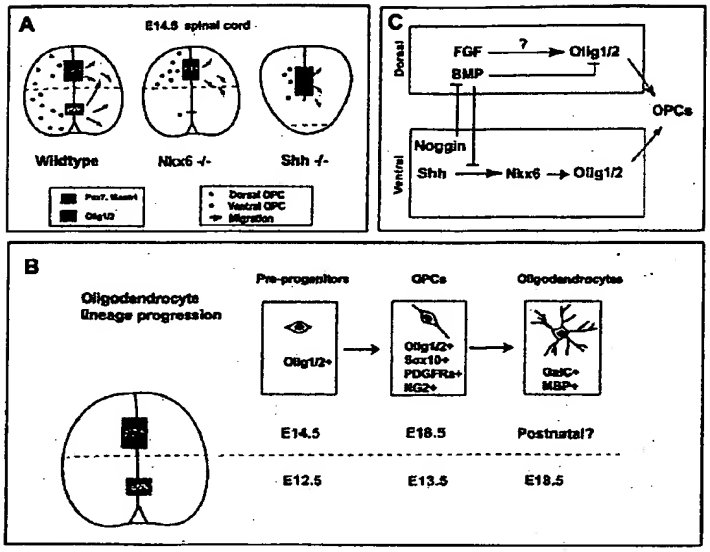

Figure 9. Proposed Origins and Molecular Specification of Oligodendrocytes in the Spinal Cord

(A) Proposed oligodendrocyte development in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutant spinal cords. In the wild-type, a vast majority of OPCs are derived from the ventral pMN domain. A subpopulation of OPCs is also generated from the dorsal dI3–dI5 domains independent of *Nkx6* and *Shh* activities. The arrows represent the possible migration directions.

(B) Time schedule of lineage progression for both dorsal- and ventral-derived OPCs. The generation of dorsal OPCs is about 2 days later than that of ventral OPCs. In general, there is a parallel delay in dorsal OPC generation and their differentiation.

(C) Proposed molecular pathways for dorsal and ventral oligodendrogenesis. *Shh* and *BMPs* are known to be the major inducer and repressor of oligodendrogenesis, respectively. In the ventral spinal cord, OPCs are generated in a *Shh/Nkx6*-dependent mechanism. Repression of *BMP* signaling by the notochord-derived Noggin may also contribute to ventral oligodendrogenesis. In the dorsal spinal cord, OPCs are generated independent of the *Shh/Nkx6* pathway and may result from a combination of FGFs and progressive loss of BMP inhibition over time.

E18.5, OPCs in both mutants could mature into *MBP*+ oligodendrocytes (Figures 4F and 8P) or even myelinate axons (Figure 4H) if they were allowed to develop further in vitro. Consistently, OPCs generated in the dorsal explants of normal embryos could also differentiate into mature oligodendrocytes (Figure 6). In general, there appears to be a parallel delay of OPC generation and their terminal differentiation as observed in other genetic mutants (Qi et al., 2003; Liu et al., 2003).

A *Shh*-Independent Pathway for Oligodendrogenesis in the Developing Spinal Cord

Early studies demonstrated that blockade of *Shh* signaling can inhibit oligodendrogenesis both in vivo and in vitro (Orentas et al., 1999; Davies and Miller, 2001; Tekki-Kessaris et al., 2001). Thus, it has been believed that *Shh* signaling is required for the development of oligodendrocytes in the entire CNS. However, the observations that OPCs can be produced from dorsal spinal cord explants in the presence of anti-*Shh* antibody (Figure 7) or from dissociated dorsal neural progenitor cells in the presence of bFGF and cyclopamine (Chandran et al., 2003; Kessaris et al., 2004) have suggested a *Shh*-independent pathway for oligodendrogenesis. However, the efficiency and specificity of the antibody and cyclopamine inhibition could potentially lead to alternative explanations. Our observation that GalC+ oligodendrocytes can develop from *Smo*$^{-/-}$ mutant ES cells provides unambiguous genetic evidence that oligodendrogenesis can occur in the absence of hedgehog signaling (Figure 7), at least in vitro.

Despite the in vitro data for *Shh*-independent oligodendrogenesis, there has been no evidence that this phenomenon can be applied to in vivo development. Our findings that OPCs are generated from *Shh*$^{-/-}$ spinal cord provide the missing link that *Shh*-independent oligodendrogenesis also occurs during spinal cord development as well. In the absence of *Shh* signaling, *Olig1/2*+ OPCs were still generated on schedule (at E14.5) as in the wild-type dorsal spinal cord. Although we can not formally exclude the possibility that the hedgehog signaling in *Shh*$^{-/-}$ mutants could be compensated by the upregulation of expression of other hedgehog members such as *Indian hedgehog* (*Ihh*) or *Desert hedgehog* (*Dhh*), we do not favor this possibility, as we failed to detect by in situ hybridization (ISH) the expression of *Ihh* or *Dhh* in either wild-type or *Shh* mutant spinal cords around the onset of dorsal oligodendrogenesis (data not shown).

The signaling mechanism underlying the *Shh*-independent late phase of dorsal oligodendrogenesis in the spinal cord is uncertain at this stage. Since bFGF can induce oligodendrocytes in dissociated dorsal neural progenitor cells (Gabay et al., 2003) independent of *Shh* signaling (Chandran et al., 2003; Kessaris et al., 2004) and in dorsal explants (our unpublished data), it is conceivable that *FGF* signaling may be partially responsible for the late production of OPCs in the dorsal spinal cord (Figure 9C). In addition, the progressive reduction of *BMP* signaling over time may also contribute to dorsal oligodendrogenesis. It is known that BMP can antagonize *Shh*-induced oligodendrocyte specification, and experimental inhibition of *BMP* signaling is sufficient to induce oligodendrocyte production both in vivo and in vitro (Mekki-Dauriac et al., 2002; Miller et al., 2004; Vallstedt et al., 2005 [this issue of *Neuron*)]. Future studies on the expression and function of various FGF and BMP molecules and their receptors will be needed to determine their possible in vivo roles in the late phase of oligodendrogenesis in the dorsal neural progenitor cells.

Multiple Origins and Phases of Oligodendrogenesis in the Developing Spinal Cord

It is generally accepted that early OPCs are induced from the pMN domain of ventral spinal cord by the *Shh* signal (Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999) and that oligodendrocyte development is coupled to motor neuron development (Richardson et al., 2000; Lu et al., 2002; Zhou and Anderson, 2002). However, our data in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutants and in wild-type embryos as well have provided strong evidence that a subset of OPCs originate from the dorsal spinal cord independent of motor neuron development at later stages of oligodendrogenesis. Therefore, there are multiple origins of, and distinct inductive mechanisms for, OPC production in the developing mammalian spinal cord. Based on these observations, we propose that there are two phases of *Olig* gene expression during normal spinal cord development, the *Shh/Nkx6*-dependent early phase of *Olig* expression and oligodendrogenesis in the pMN domain and the *Shh/Nkx6*-independent late phase of *Olig* expression and oligodendrogenesis in the dorsal spinal cord (Figures 9A and 9B).

The dorsal oligodendrogenesis in mouse spinal cord has long been unnoticed, because the production of OPC cells from the dorsal spinal cord is both late and transient (at around E14.5) as compared to the early OPC production (E12.5) from the ventral spinal cord. By the time OPCs are being generated from the dorsal spinal cord, a large number of ventral-derived OPCs have already invaded into the dorsal spinal cord and thus mask the existence of the late-born dorsal OPCs. Only in mutants (e.g., *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$) in which the ventral oligodendrogenesis from the pMN domain is inhibited or greatly compromised can the dorsal generation of OPCs be uncovered.

Experimental Procedures

Genotyping of *Nkx6* Mutant Mice

The *Nkx6.1* and *Nkx6.2* homozygous null embryos were obtained by the interbreeding of double heterozygous animals. Genomic DNA extracted from embryonic tissues or mouse tails was used for genotyping by Southern analysis or by PCR. Genotyping of *Nkx6.1* and *Nkx6.2* loci was described in Sander et al. (2000) and Cai et al. (2001), respectively. Genotyping of *Shh* mutant mice was carried out according to Chiang et al. (1996).

In Situ RNA Hybridization and Immunohistochemical Staining

Spinal cord tissues at the thoracic level were isolated from E10.5 to E18.5 mouse embryos and then fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media, and then sectioned (20 μm thickness) on a cryostat. Adjacent sections from the wild-type and mutant embryos were subsequently subjected to ISH or immunofluorescent staining. ISH was performed as described in Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications, and the detailed protocol is available upon request. Double immunofluorescent procedures were previously described in Xu et al. (2000). For the combination of ISH and immunohistochemistry, sections were first subject to ISH with *Dbx2* riboprobe, rinsed several times with PBS followed by immunohistochemical staining with anti-*Olig2*. Anti-*Pax7* (1:50 DSHB), anti-*Mash1* (1:200), anti-NG2 (1:1500), anti-*PDGFRα* (1:300), anti-*MBP* (1:5000), and anti-GalC (1:50) were obtained from commercial sources. Anti-*Olig2* (1:3000) and anti-*Sox10* (1:3000) polyclonal antibodies were generously provided by Drs. Chuck Stiles and Michael Wegner.

Spinal Cord Explant Culture

Segments of spinal cord tissues were isolated from E11.5, E13.5, or E18.5 embryos at the thoracic region and grown either in collagen gel or on 8.0 μm nucleopore polycarbonate membranes (Costar) floating on culture medium (DMEM + N2 supplement + 30 ng/ml T3 + 40 ng/ml T4 + 1 mg/ml BSA + 0.5% FBS + Pen-Strep). In our experience, the inclusion of a small amount of FBS in culture medium made cells healthier and did not appear to significantly affect oligodendrocyte development in explant culture as compared to no serum (data not shown), although serum was shown to inhibit OPC differentiation (Raff et al., 1983). For anti-*Shh* antibody treatment, 5E1 supernatant (1:3 DSHB) was added to culture medium. Following various days of culture in vitro, explants were then fixed in 4% PFA and processed for immunofluorescent staining (Xu et al., 2000) or whole-mount in situ RNA hybridization with *MBP* riboprobe, as described in Cai et al. (1999).

Culture and Differentiation of Embryonic Stem Cells

Normal and *Smo*$^{-/-}$ ES cells were maintained on MEF feeder cells in ES medium with LIF. During differentiation, ES cells were dissociated and grown on nonadherent petri dishes for 2 days in the absence of LIF and 4 additional days in 5 μM retinoic acid to generate embryoid bodies (EB). Following 10 days of suspension culture, EBs were trypsinized with trypsin/EDTA, and cells were plated on laminin-coated cover slips and cultured for 15 days prior to immunofluorescent staining with anti-GalC antibody.

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Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains

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SUMMARY

The postnatal central nervous system (CNS) contains many scattered cells that express fibroblast growth factor receptor 3 transcripts (*Fgfr3*). They first appear in the ventricular zone (VZ) of the embryonic spinal cord in mid-gestation and then distribute into both grey and white matter – suggesting that they are glial cells, not neurones. The *Fgfr3*$^+$ cells are interspersed with but distinct from platelet-derived growth factor receptor α (*Pdgfra*)-positive oligodendrocyte progenitors. This fits with the observation that *Fgfr3* expression is preferentially excluded from the pMN domain of the ventral VZ where *Pdgfra*$^+$ oligodendrocyte progenitors – and motoneurones – originate. Many glial fibrillary acidic protein (Gfap)-positive astrocytes co-express *Fgfr3* in vitro and in vivo. *Fgfr3*$^+$ cells within and outside the VZ also express the astroglial marker glutamine synthetase (*Glns*). We conclude that (1) *Fgfr3* marks astrocytes and their neuroepithelial precursors in the developing CNS and (2) astrocytes and oligodendrocytes originate in complementary domains of the VZ. Production of astrocytes from cultured neuroepithelial cells is hedgehog independent, whereas oligodendrocyte development requires hedgehog signalling, adding further support to the idea that astrocytes and oligodendrocytes can develop independently. In addition, we found that mice with a targeted deletion in the *Fgfr3* locus strongly upregulate Gfap in grey matter (protoplasmic) astrocytes, implying that signalling through Fgfr3 normally represses Gfap expression in vivo.

Key words: Fgfr3, Targeted deletion, Astrocyte, Reactive gliosis, CNS, Neuroepithelium

INTRODUCTION

In the embryonic CNS, neurones and glia develop from the neuroepithelial cells of the ventricular zone (VZ) that surrounds the ventricles of the brain and the lumen of the spinal cord. Different domains of the VZ express different gene products and generate different subsets of neurones and/or glia. For example, the ventral half of the spinal cord VZ is subdivided into five regions labelled (from ventral to dorsal) p3, pMN, p2, p1 and p0. These five domains express different combinations of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors and generate distinct classes of spinal neurones; pMN gives rise to somatic motoneurones, whereas p0-p3 give rise to four classes of ventral interneurones (V0-V3 respectively) (reviewed by Briscoe and Ericson, 1999; Jessell, 2001). In the brainstem, p3 also gives rise to visceral motoneurones (Ericson et al., 1997).

After neurones, the VZ switches to producing glial cells. Oligodendrocytes, the myelinating glial cells of the CNS, develop from the ventral VZ. Small numbers of oligodendrocyte progenitors (OLPs), which express the platelet-derived growth factor receptor-α (Pdgfra), first appear at the ventricular surface on embryonic day 12.5 (E12.5) in the mouse, then proliferate and migrate away into the grey and white matter before starting to differentiate into myelin-forming oligodendrocytes (Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). In rodents, OLPs are generated from the same part of the neuroepithelium as somatic motoneurones (MNs) but not until after MN production has ceased (Sun et al., 1998; Lu et al., 2000) (for a review, see Rowitch et al., 2002). This prompted us to suggest that there is a pool of shared neuroglial precursors that first generates MNs, then switches to OLPs (Richardson et al., 1997; Richardson et al., 2000). This idea has been supported recently by the finding that the bHLH proteins Olig1 and Olig2 are expressed and required in pMN for production of both motoneurones and OLPs (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002) (reviewed by Rowitch et al., 2002).

Where do astrocytes, the other major class of CNS glia, originate in the neuroepithelium? It is believed that at least some astrocytes are generated by transdifferentiation of radial

glia (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). Others are formed from multipotent precursors in the subventricular zones (SVZ) of the postnatal brain. However, the origins of astrocytes in the developing spinal cord are unclear, so we looked for an astrocyte lineage marker that might be helpful in following the development of astrocytes from their earliest precursors in the VZ. Previous expression studies of the fibroblast growth factor receptor 3 (*Fgfr3*) suggested that this receptor might be expressed in glial cells, possibly astrocytes (Peters et al., 1993; Miyake et al., 1996). Our own studies, reported here, support this conclusion and suggest that *Fgfr3*-positive astrocytes develop from *Fgfr3*-positive precursor cells in the neuroepithelium. *Fgfr3* is not expressed equally in all parts of the neuroepithelium but is reduced or absent from pMN, suggesting that astrocytes and OLPs have separate neuroepithelial origins. We also found that astrocytes are formed in vitro in the absence of hedgehog signalling – unlike oligodendrocytes, which require sonic hedgehog from the ventral midline. This reinforces the notion that at least some astrocytes develop independently of OLPs.

To investigate the function of *Fgfr3* in astrocytes, we examined mice with a targeted deletion in the *Fgfr3* locus (Colvin et al., 1996). The number of *Fgfr3*-expressing cells was normal in the knockout, suggesting that *Fgfr3* does not mediate a mitogenic or survival-promoting effect for these cells. However, Gfap was markedly upregulated in grey matter astrocytes, which normally have little or no Gfap – unlike their counterparts in white matter. Our results imply that signalling through Fgfr3 normally represses Gfap expression in grey matter astrocytes and suggest that white matter astrocytes might preferentially express Gfap because ligands for Fgfr3 are not normally available in axon tracts.

MATERIALS AND METHODS

Tissue and cell cultures

Spinal cords from stage 12-13 (48 hour) chick embryos were dissected into dorsal, middle and ventral thirds using a flame-sharpened tungsten needle. Tissue fragments were cultured as explants in collagen gels (Guthrie and Lumsden, 1994) in defined BS medium (Bottenstein and Sato, 1979) containing 0.25% (v/v) foetal bovine serum (FBS) and conalbumin in place of transferrin (Pringle et al., 1996).

For dissociated cell cultures, E17 rat cervical spinal cords were digested in 0.25% (w/v) trypsin in Earle's buffered saline (Ca^{2+} and Mg^{2+} free; Gibco) for 15 minutes at 37°C, then FBS was added to a final concentration of 10% (v/v) and the tissue physically dissociated by trituration. Cells were washed by centrifugation and resuspended in BS medium before plating in a 50 µl droplet on poly-D-lysine-coated glass coverslips (5×10^4 cells/coverslip). Both explants and dissociated cell cultures were cultured at 37°C in 5% CO_2 in a humidified atmosphere.

Neutralising Shh activity in vitro

Monoclonal Shh neutralising antibody 5E1 (Ericson et al., 1996) was concentrated by ammonium sulphate precipitation from hybridoma supernatants (Harlow and Lane, 1988). Monoclonal anti-NG2 proteoglycan #4.11 (Stallcup and Beasley, 1987) was used as a negative control. Precipitated antibodies were dissolved in a small volume of PBS and dialysed first against PBS and then Dulbecco's modified Eagle's medium (DMEM, Gibco). The final volumes were approximately tenfold less than the starting volumes and were assumed to be ten time as concentrated. Explants were incubated in the presence of either anti-Shh or control antibodies at twice the final concentration. Antibodies were added at the start of the experiment and fresh medium and antibody were added each day thereafter. In some experiments cyclopamine (1 µM; from William Gaffield) instead of anti-Shh was added to cultures daily.

BrdU labelling in vivo

E18 pregnant mice were injected intraperitoneally with BrdU at 50 µg BrdU per gram bodyweight. Two injections were given, 6 hours apart. Mice were sacrificed 3 hours after the second injection and the embryos were processed for *Fgfr3* in situ hybridisation and BrdU immunolabelling.

Preparation of tissue sections

C57Bl/6 mice were obtained from Olac and bred in-house. Noon on the day of discovery of the vaginal plug was designated embryonic day 0.5 (E0.5). We also used *Fgfr3*-null mice (Colvin et al., 1996) bred at UCL. Mid-gestation embryos were staged according to the morphological criteria of Theiler (Theiler, 1972). Rats (Sprague-Dawley) were obtained from the UCL breeding colony and staged according to Long and Burlingame (Long and Burlingame, 1938). Fertilised White Leghorn chicken eggs were obtained from Needle Farm (Cambridge, UK). They were incubated at 38°C and the chicken embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Embryos were decapitated and immersion-fixed in cold 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours before cryoprotecting in cold 20% (w/v) sucrose in PBS for at least 24 hours. In sections processed for immunohistochemistry after in situ hybridisation, the fixation time was reduced to 1 hour to preserve epitope integrity. Tissues were immersed in OCT embedding compound (BDH), frozen on solid CO_2 and stored at –70°C before sectioning. Frozen sections (15 µm) were cut on a cryostat and collected on 3-aminopropyl-triethoxysilane (APES)-coated glass microscope slides. Sections were air-dried for 2 hours before storing dry at –70°C.

Immunohistochemistry

Anti-Gfap monoclonal ascites, clone G-A-5 (Sigma), was used at a dilution of 1:400. Anti-BrdU (monoclonal BU209) (Magaud et al., 1989) was used at 1:5 dilution. Monoclonal O4 (Sommer and Schachner, 1981) was used as cell culture supernatant diluted 1:5. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (all from Pierce) diluted 1:200. All antibodies were diluted in PBS containing 0.1% (v/v) Triton X-100 and 10% (v/v) normal goat serum, except O4, which was diluted in PBS alone. Sometimes diaminobenzidine (DAB) labelling (ABC kit, Vector Laboratories) was used instead of fluorescence detection.

In situ hybridisation

Our in situ hybridisation procedures have been described (Pringle et al., 1996; Fruttiger et al., 1999); detailed protocols are available at http://www.ucl.ac.uk/~ucbzwdr/richardson.htm. Digoxigenin (DIG)- or fluorescein (FITC)-labelled RNA probes were transcribed in vitro from cloned cDNAs. The rat *Fgfr3* probe was transcribed from a ~900 bp partial cDNA encoding most of the tyrosine kinase (TK) domain (W.-P. Yu, PhD thesis, University of London, 1995) and the chicken *Fgfr3* probe from a ~440 bp partial cDNA encoding part of the TK domain (from Ivor Mason, King's College London). The mouse *Pdgfra* probe was made from a ~1600 bp cDNA encoding most of the extracellular domain (from Chiayeng Wang, University of Chicago). The chicken *Pdgfra* probe was made from a ~3200 bp cDNA covering most of the 3′ untranslated region of the mRNA (from Marc Mercola, Harvard Medical School, Boston).

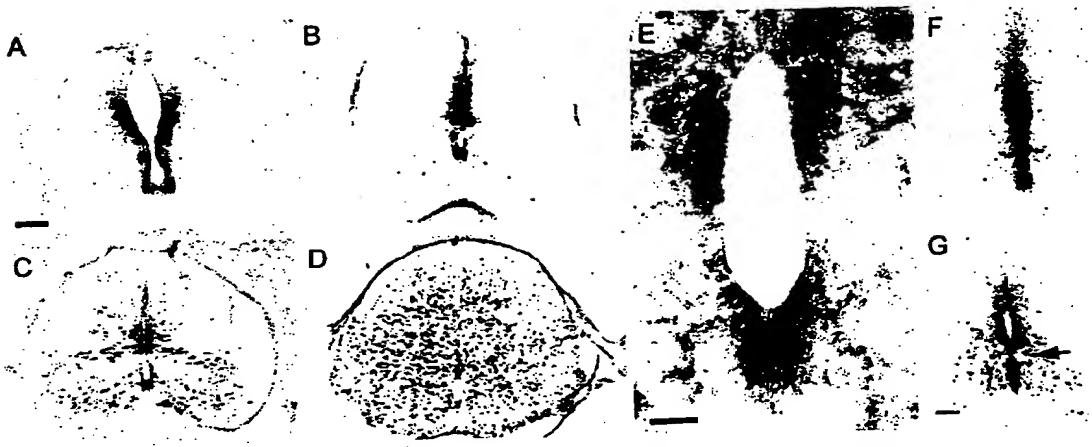

Fig. 1. *Fgfr3* expression in transverse sections of embryonic chick and mouse cervical spinal cords. (A) Chick stage 22-24 (E3.5-4); (B) chick stage 34 (E8); (C) chick stage 35 (E9); (D) chick stage 37 (E11); (E) chick stage 35 (E9); (F) mouse E13.5; and (G) mouse E14.5. Initially, *Fgfr3* is expressed in the floor plate and the ventral two-thirds of the VZ (A) and is later downregulated in part of the ventral VZ (B). Starting around stage 34 (E8) *Fgfr3*$^+$ cells are visible in the parenchyma of the cord. By stage 37 (E11) the floor plate and VZ no longer express *Fgfr3* but scattered *Fgfr3*$^+$ cells are present throughout most of the cross-section of the cord, including both grey and white matter (D). (E) A magnified image of the ventral VZ from a stage 35 (E9) cord, showing the two spatially separated domains of *Fgfr3* expression. A similar progression occurs in mouse (F,G). However, the ventral 'gap' is not so pronounced in mouse (arrow in G). Scale bars: 200 μm (A-D), 100 μm (F,G), 50 μm (E).

For double in situ hybridisation, two probes – one FITC labelled and the other DIG labelled – were applied to sections simultaneously. The FITC signal was visualised with alkaline phosphatase (AP)-conjugated anti-FITC Fab_2 fragments before developing in p-iodonitrotetrazolium violet (INT) and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (BCIP), which produces a magenta/brown reaction product. The sections were photographed, then the AP was inactivated by heating at 65°C for 30 minutes followed by incubating in 0.2 M glycine (pH 2) for 30 minutes at room temperature. The INT-BCIP reaction product was removed by dehydration through graded alcohols, concluding with 100% ethanol for 10 minutes at room temperature. The DIG signal was then visualised with AP-conjugated anti-DIG Fab_2 fragments and a mixture of nitroblue tetrazolium (NBT) and BCIP (all reagents from Roche Molecular Biochemicals) and the sections re-photographed. No labelling with NBT/BCIP was observed when we omitted either the DIG labelled probe or the anti-DIG antibody (data not shown).

For the *Fgfr3-Pdgfra* double in situ hybridisation of Fig. 4 we visualised the FITC (*Pdgfra*) signal with horseradish peroxidase (HRP)-conjugated anti-FITC Fab_2 fragments (Roche) before developing in fluorescein-tyramide reagent (NEN™ Life Science Products, Boston) according to the manufacturer's instructions. The HRP-conjugate was inactivated by incubating in 2% (v/v) hydrogen peroxide for 30 minutes at room temperature. The DIG (*Fgfr3*) signal was then visualised with HRP-conjugated anti-DIG Fab_2 fragments followed by rhodamine-tyramide, and the sections photographed under fluorescence optics. As specificity controls we omitted either the FITC-labelled *Pdgfra* probe or the HRP-conjugated anti-FITC antibody, which gave no staining other than for *Fgfr3* (not shown).

Combined immunolabelling and in situ hybridisation

For the experiment of Fig. 7, cultured cells were first subjected to in situ hybridisation with a [^{35}S]-labelled RNA probe against *Fgfr3* then immunolabeled with anti-Gfap and biotinylated goat-anti-mouse Ig. The Gfap signal was developed with DAB and the slides dehydrated through ascending alcohols, dipped in nuclear emulsion (Ilford K5), exposed in the dark for several days and developed in Kodak D19.

RESULTS

Fgfr3 expression in the embryonic spinal cord

We examined *Fgfr3* expression in the embryonic chick spinal cord by in situ hybridisation. At stage 22-24 (corresponding to ~E4), *Fgfr3* expression was confined to the floor plate and the ventral two-thirds of the VZ (Fig. 1A). By stage 34 (E8) *Fgfr3* expression had been extinguished in part of the ventral VZ so that a gap developed in the expression pattern (e.g. Fig. 1B).

Individual *Fgfr3*$^+$ cells were also present outside the VZ

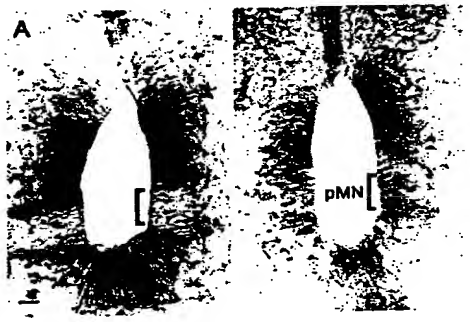

Fig. 2. Expression of *Fgfr3* and *Olig2*. Transverse sections through stage 35 (E9) chicken spinal cords were subjected to in situ hybridisation for *Fgfr3* (A) or double in situ for *Fgfr3* and *Olig2* (B). At this age, *Fgfr3* expression is confined to the VZ and a few scattered cells outside the VZ. The two spatially separated domains of *Fgfr3* expression are clearly visible (A). *Olig2* is expressed predominantly within the ventral 'gap' of *Fgfr3* expression (B). This suggests that pMN (brackets), which generates *Pdgfra*$^+$ oligodendrocyte progenitors (OLPs), does not also generate *Fgfr3*$^+$ putative astrocyte progenitors. Scale bar: 50 μm.

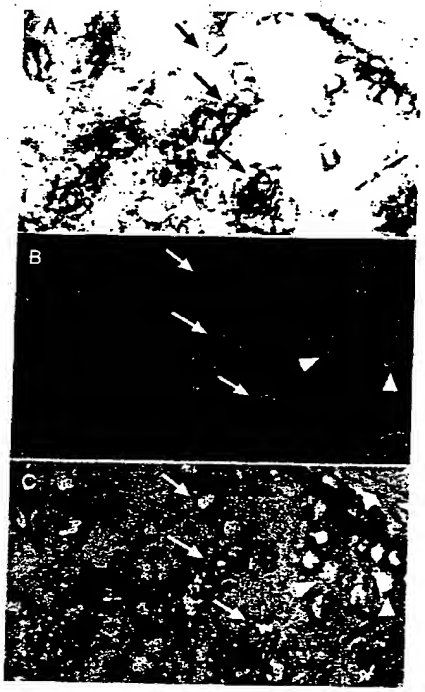

Fig. 3. Incorporation of BrdU by *Fgfr3*-expressing cells. We labelled E18 embryos by two intra-peritoneal injections of BrdU, 6 hours apart, into the mother. We harvested the embryos 3 hours later and performed in situ hybridisation for *Fgfr3* followed by immunohistochemistry for BrdU. The *Fgfr3* (A) and BrdU (B) images were superimposed using Adobe Photoshop (C). Many *Fgfr3*-expressing cells incorporated BrdU (C, arrows), confirming that they can divide after exiting the VZ and are therefore unlikely to be neurones. Arrowheads in B,C indicate *Fgfr3*-negative cells that have incorporated BrdU.

after stage 34 (E8), both lateral and dorsal to the *Fgfr3*$^+$ neuroepithelial domains. Often the individual cells appeared to be streaming away from the VZ into the parenchyma. This is evident in Fig. 1C, for example. By stage 37 (E11) *Fgfr3* expression was no longer detectable in the VZ but scattered *Fgfr3*$^+$ cells were present throughout the grey and white matter of the cord (Fig. 1D). *Fgfr3* expression followed a similar progression in mouse and rat (Fig. 1F,G and not shown). In rodents, however, the ventral gap in *Fgfr3* expression was not as pronounced as in chicks (Fig. 1G, arrow).

A scattered population of *Fgfr3*-expressing cells is found throughout most regions of the late embryonic and postnatal mouse brain, both in white and in grey matter. As in the embryonic spinal cord, there appear to be specific regions of the embryonic brain VZ that give rise to *Fgfr3*$^+$ cells that stream away from the VZ into the parenchyma (not shown).

Fgfr3-expressing cells originate mainly outside the pMN domain of the neuroepithelium

In the developing spinal cord, neuroepithelial precursors at different positions along the dorsoventral axis generate distinct neuronal subtypes. The ventral half of the spinal cord VZ is

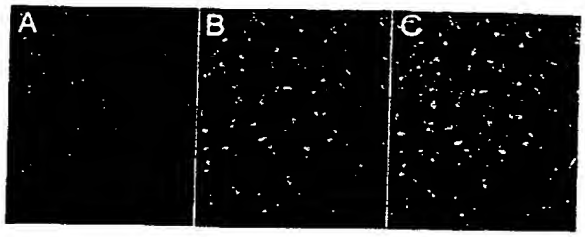

Fig. 4. Different populations of *Fgfr3*$^+$ and *Pdgfra*$^+$ cells in the newborn spinal cord. We hybridised sections of P2 mouse cervical spinal cord simultaneously with a DIG-labelled *Fgfr3* probe together with an FITC-labelled *Pdgfra* probe to visualise OLPs. The *Fgfr3* signal (red) was visualised with rhodamine-tyramide reagent and the *Pdgfra* signal (green) with fluorescein-tyramide. Scattered individual *Fgfr3*$^+$ and *Pdgfra*$^+$ cells can be seen throughout both white and grey matter of the cord, but these are separate and discrete cell populations. We conclude that the great majority of *Fgfr3*$^+$ cells in the cord are not OLPs.

divided into five neuroepithelial domains known as (from ventral to dorsal) p3, pMN, p2, p1 and p0 (Briscoe et al., 2000). Of these, pMN is known to generate motoneurones followed by oligodendrocyte progenitors (OLPs). It seemed to us that the ventral gap in *Fgfr3* expression (Fig. 2A) might correspond to pMN. To test this, we performed double in situ hybridisation for *Fgfr3* and *Olig2* (which defines pMN) (Lu et al., 2000; Zhou et al., 2000). At stage 35, the *Olig2* in situ hybridisation signal was within the gap in the *Fgfr3* signal (Fig. 2B, arrow). Therefore, *Fgfr3* is preferentially downregulated in pMN where oligodendrocyte lineage cells originate, but is expressed both ventral and dorsal to pMN.

Fgfr3- expressing cells are glia

The fact that most of the scattered *Fgfr3*$^+$ cells are generated after stage 34 (E8) in the chick, E13.5 in mouse, is itself a strong argument that they are glial cells, not neurones, because most spinal neurones are born before this (Altman and Bayer, 1984). That some of the *Fgfr3*$^+$ cells are found in axon tracts also suggests that they are glia, for there are very few neuronal cell bodies in fibre tracts.

Another indication that they are glial cells is that they continue to divide after they leave the VZ. We showed this by injecting BrdU into a pregnant mouse at18 days gestation. The embryos were removed 3 hours later and processed by in situ hybridisation for *Fgfr3* followed by immunolabelling for BrdU. We found many (*Fgfr3*$^+$, BrdU$^+$) cells scattered throughout the white and grey matter of the cord (Fig. 3, arrows). This confirms that *Fgfr3*-expressing cells divide in vivo and are therefore unlikely to be neurones or neuronal progenitors, which leave the VZ as postmitotic cells. This strengthens the idea that the *Fgfr3*-expressing cells are glia. There was also a population of (BrdU$^+$, *Fgfr3*$^-$) cells in both grey and white matter (Fig. 3C, arrowheads), so there is a distinct population(s) of dividing cells that do not express *Fgfr3*.

Fgfr3-expressing cells are distinct from Pdgfra$^+$ oligodendrocyte progenitors

To determine whether the *Fgfr3*$^+$ cells that we detect are oligodendrocyte progenitors (OLPs), we double labelled

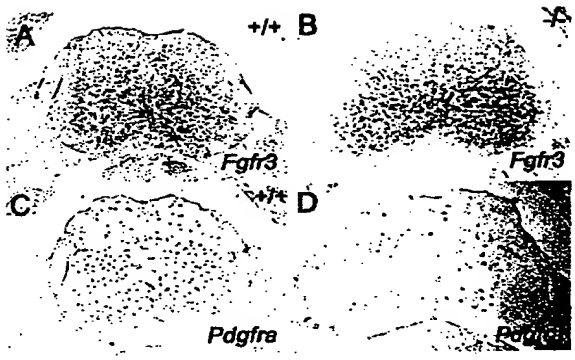

Fig. 5. *Fgfr3*-positive cells are unaffected in *Pdgfa* null spinal cords. Consecutive sections of newborn wild-type or *Pdgfa* knockout mouse cervical spinal cords were hybridised in situ with probes to *Fgfr3* (A,B) or *Pdgfra* (C,D). The number of *Pdgfra*$^+$ OLPs is strongly reduced in the *Pdgf-A* knockout (compare C with D) but neither the number nor the distribution of *Fgfr3*$^+$ cells is changed noticeably (A,B). Again, we conclude that the *Fgfr3*$^+$ cells and *Pdgfra*$^+$ OLPs are different cells.

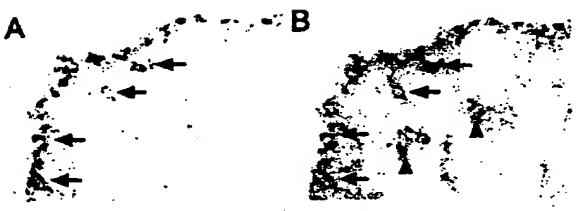

Fig. 6. Newly differentiating white matter astrocytes express *Fgfr3*. We simultaneously hybridised sections of E18 mouse cervical spinal cord with an FITC-labelled *Gfap* mRNA probe (A) and a DIG-labelled *Fgfr3* probe (B). The *Gfap* and *Fgfr3* hybridisation signals were visualised and photographed sequentially (see Materials and Methods). All the *Gfap*-expressing astrocytes also expressed *Fgfr3* (e.g. arrows). In general, *Fgfr3*$^+$ cells in the grey matter (arrowheads) did not co-express *Gfap*.

mouse E18 and P2 spinal cord sections for *Fgfr3* and *Pdgfra*, an established marker of early OLPs. Both in situ hybridisation probes labelled similar numbers of cells that were scattered throughout the spinal cord grey and white matter, but the two cell populations were completely non-overlapping (Fig. 4). This also held true throughout the postnatal brain (N. P. P., unpublished). We also looked in newborn *Pdgfa* knockout mice which contain far fewer *Pdgfra*$^+$ OLPs than normal (Fruttiger et al., 1999). Despite the lack of OLPs, there were normal numbers of *Fgfr3*$^+$ cells at this age (Fig. 5). Clearly, the *Fgfr3*$^+$ cells detected by our in situ hybridisation procedures are not early OLPs but a different cell population. This is consistent with the fact that in mice lacking Fgfr3, early events of oligodendrocyte lineage progression occur normally and the numbers of *Pdgfra*$^+$ cells remains unchanged (R. Bansal, personal communication) (N. P. P., unpublished).

Fgfr3-expressing cells are astrocytes and astrocyte precursors

To test whether the *Fgfr3*$^+$ cells might be astrocytes, we double labelled E18 mouse spinal cord sections for *Fgfr3* and *Gfap* mRNAs. At E18, white matter astrocytes begin to express *Gfap* mRNA, which initially remains in the astrocyte cell bodies and allows identification of individual astrocytes. (As astrocytes mature further, both *Gfap* mRNA and protein are relocated to the extending cell processes, making individual cells difficult to distinguish.)

All the *Gfap*$^+$ astrocytes in developing white matter at E18 also expressed *Fgfr3* (Fig. 6, arrows). This result clearly identifies many of the *Fgfr3*-expressing cells as astrocytes. Nevertheless, the majority of *Fgfr3*-expressing cells in the grey matter (Fig. 6B, arrowheads) are *Gfap*-negative. We presume that these represent *Gfap*-negative, possibly immature, astrocytes.

In an attempt to label all astrocytes, including *Gfap*-negative astrocytes, we used an in situ hybridisation probe against glutamine synthetase mRNA (*Glns*) (EC 6.3.1.2). Glns is widely regarded as an astrocyte marker, although there have been reports that it is also present in mature oligodendrocytes and even OLPs. We found that *Glns* transcripts were present in the VZ of the E15 mouse spinal cord and in cells outside the VZ in a pattern that was very similar that of *Fgfr3* (Fig. 7). This is consistent with the view that *Fgfr3* and *Glns* mark astrocytes and their precursors. This conclusion was further strengthened by studies of cultured astrocytes (see below).

Cultured astrocytes co-express Gfap and Fgfr3

When CNS cells are dissociated and placed in culture, astrocytes in the culture upregulate Gfap and are easily recognisable. We dissociated and cultured cells from E17 rat cervical spinal cord and labelled them by in situ hybridisation for *Fgfr3* and by immunocytochemistry for Gfap. Almost all of the Gfap$^+$ astrocytes also expressed *Fgfr3* (Table 1; Fig. 8, arrows). There was also a small population of flat, fibroblast-like *Fgfr3*$^+$ cells that did not express Gfap (Fig. 8, arrowheads). The number of these cells decreased with time in culture; at 3 days they were 6% of all cells, by 9 days less than 1% (Table 1). These (*Fgfr3*$^+$, Gfap$^-$) cells might be astrocyte precursors or immature astrocytes that have not yet upregulated Gfap. In any

Table 1. E17 rat spinal cord cell cultures double labelled for *Fgfr3* and Gfap

Days in vitro	*Fgfr3*$^+$ Gfap$^-$ (astrocyte precursors?)	*Fgfr3*$^+$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^-$ (other cells)
3	17/275 (6%)	69/275 (25%)	None	189/275 (68%)
6	23/596 (4%)	82/596 (14%)	2/596 (<1%)	489/596 (82%)
9	4/645 (<1%)	197/645 (31%)	1/645 (<1%)	443/645 (69%)

Dissociated cells from E17 rat spinal cord were cultured for 3, 6 or 9 days and then subjected to in situ hybridization for *Fgfr3* mRNA followed by immunohistochemistry for Gfap protein. We counted astrocytes (*Fgfr3*$^+$, Gfap$^+$ and *Fgfr3*$^-$, Gfap$^+$), putative astrocyte precursors (*Fgfr3*$^+$, Gfap$^-$) and other unidentified cells (*Fgfr3*$^-$, GFAP$^-$). The great majority of GFAP-expressing astrocytes also expressed *Fgfr3*. These data are from a single representative experiment (duplicate coverslips); comparable results were obtained in two additional independent experiments.

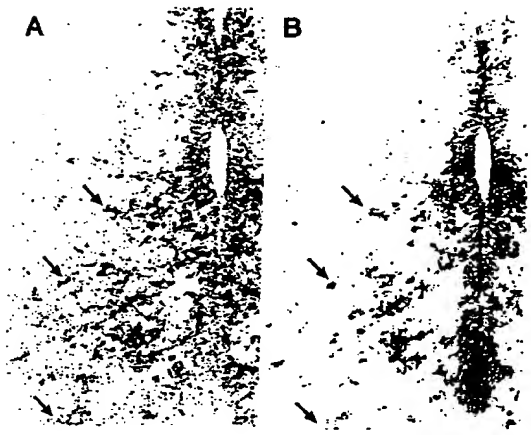

Fig. 7. Co-expression of *Fgfr3* and glutamine synthetase (*Glns*) in the VZ and parenchyma of the embryonic mouse spinal cord. There was considerable overlap between the in situ hybridisation signals for *Fgfr3* and *Glns* in the E15 mouse spinal cord, strongly suggesting that *Fgfr3*$^+$ cells correspond to glial (presumably astrocyte) precursors. Arrows indicate cells that express both *Fgfr3* and *Glns*.

case, this experiment provides clear evidence that most or all Gfap$^+$ astrocytes in culture co-express *Fgfr3*.

Gfap is upregulated in grey matter astrocytes in Fgfr3 null mice

If *Fgfr3* is expressed by astrocytes, we might expect to see specific effects on astrocytes in transgenic mice homozygous for a targeted disruption of *Fgfr3*. These mice have previously been shown to have skeletal and inner ear defects but no CNS defects have yet been reported (Colvin et al., 1996).

We visualised astrocytes in spinal cord sections of 3-month-old *Fgfr3* null mice, together with their heterozygous *Fgfr3*$^{+/-}$ and wild-type littermates, by immunolabelling with anti-Gfap. Heterozygous and null mutant mice all displayed the normal pattern of Gfap expression up to 6 weeks of age. Gfap expression was observed in the white matter around the circumference of the spinal cord, many Gfap-labelled processes being oriented in a radial direction (Fig. 9A). By comparison, there was little or no Gfap expression in the grey matter, except in astrocytes associated with blood vessels. Between 6 weeks and 2 months of age, a striking up-regulation of Gfap expression occurred in the grey matter of *Fgfr3* null mice, though not in their heterozygous or wild-type littermates (Fig. 9B). Astrocytes lining blood vessels also had increased Gfap immunoreactivity.

The number of cells that contain *Fgfr3* mRNA was not noticeably different in *Fgfr3*-null spinal cords compared with wild type (data not shown). This suggests that Fgfr3 does not normally mediate a signal for proliferation or survival of astrocytes, although further experiments (e.g. BrdU labelling in vivo) would be required to substantiate this.

Astrocyte development in vitro does not depend on Hedgehog signalling

In the spinal cord, production of ventral cell types – motoneurones, ventral interneurones and OLPs – is dependent

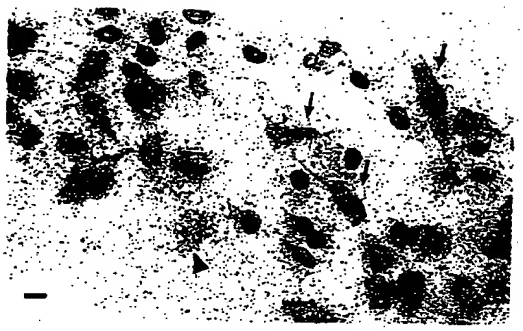

Fig. 8. Cultured cells from E17 rat spinal cord double-labelled for *Fgfr3* and Gfap. Cells were hybridised in situ with a ^{35}S-labelled RNA probe for *Fgfr3*, then immunolabelled for Gfap followed by autoradiography (see Materials and Methods). The *Fgfr3* signal (black silver grains) is present over most Gfap-positive cells (brown DAB reaction product; arrows) (also see Table 1). Scale bar: 10 μm. Arrowhead indicates an *Fgfr3*-positive, *Gfap*-negative cell.

on Shh signalling (Ericson et al., 1996; Orentas et al., 1999) (for a review, see Jessell, 2001). We wanted to know whether production of astrocytes from the ventral neural tube is also dependent on Shh. We microdissected stage 12/13 (E2) chick spinal cord into thirds along the dorsoventral axis and cultured the ventral-most fragments in collagen gels with either a control antibody or an anti-Shh neutralising antibody (see Materials and Methods). After 48 hours in culture we labelled explants with monoclonal antibody 4D5, which recognises homeodomain proteins Isl1 and Isl2 in motoneurones. Control explants contained numerous Isl$^+$ cells, whereas none were observed in explants incubated with anti-Shh (data not shown). After a further 10 days in culture (12 days total) we visualised OLPs with monoclonal antibody O4 (Sommer and Schachner, 1981) (Fig. 10C,D) and astrocytes with anti-Gfap (Fig. 10A,B). All of the explants incubated with control antibody (19/19) contained large numbers (>300) of O4$^+$ late-stage OLPs (Fig. 10C). As expected, OLP production was markedly decreased by anti-Shh (Fig. 10D); 14/22 explants contained no O4$^+$ cells and, of the remaining eight explants, seven contained fewer than ten positive cells and the other one contained 38 positive cells. By contrast, all explants contained numerous (>300) Gfap$^+$ astrocytes whether they had been incubated with control antibody (19/19) or anti-Shh (22/22) (Fig. 10A,B).

Similar results were obtained with explants from stage 25 (E5) embryos from which we were able to dissect the ventral one-quarter of the neural tube and discard the floor plate. Once again, large numbers of Gfap$^+$ astrocytes developed in explants cultured with control antibody (22/22) and with anti-Shh (25/25), even though OLP production in these explants was inhibited by anti-Shh (not shown).

To test the possibility that other hedgehog (Hh) proteins (Desert Hh, Indian Hh) control astrocyte production in ventral explants, we inhibited the activity of all isoforms with the alkaloid cyclopamine (Cooper et al., 1998; Incardona et al., 1998). This gave similar results as Shh neutralising antibodies (data not shown). Thus, we conclude that astrocyte induction in ventral spinal cord does not require Hedgehog signalling.

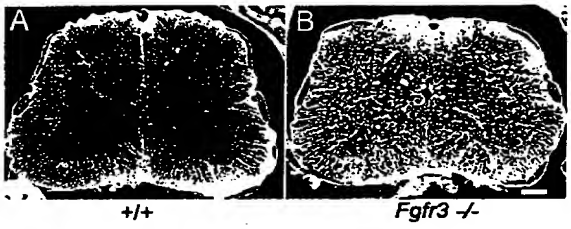

Fig. 9. Gfap upregulation in grey matter astrocytes in *Fgfr3*-null mice. Transverse sections through the cervical spinal cords of 2-month-old wild-type (A) and *Fgfr3*-null mice (B) were immunolabeled with anti-Gfap. In the wild-type cord, white matter (fibrous) astrocytes express Gfap but there is little or no Gfap immunoreactivity in the grey matter. By contrast, the *Fgfr3*-null mouse (B) shows extensive Gfap labelling of grey matter (protoplasmic) astrocytes. Scale bar: 100 μm.

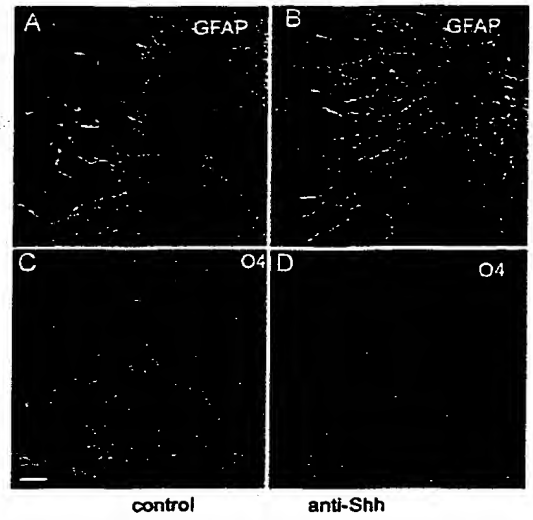

Fig. 10. Neutralising *Shh* activity in explant cultures of ventral spinal cord. Stage 12/13 (E2) chick neural tube was dissected into dorsal, intermediate and ventral thirds. The ventral thirds were cultured in collagen gels in the presence of either control antibody (A,C) or with neutralising anti-Shh antibody (B,D). Explants were double-labelled with O4 monoclonal antibody (C,D) and anti-Gfap (A,B). Anti-Shh blocks the formation of O4-positive OLPs but not Gfap-positive astrocytes. Scale bar: 10 μm.

DISCUSSION

On the basis of their spatial distribution and time of appearance, Peters et al. (Peters et al., 1993) suggested that *Fgfr3*$^+$ cells in the mouse CNS are glial cells, possibly astrocytes. By double labelling experiments with *Fgfr3* and Gfap, Miyake et al. (Miyake et al., 1996) concluded that *Fgfr3* was expressed in astrocytes in the adult rat brain. Our data support and extend these conclusions. We present evidence that scattered *Fgfr3*$^+$cells in the embryonic and postnatal CNS are astrocytes and/or astrocyte progenitors, and that these astrocytes are derived from *Fgfr3*$^+$ neuroepithelial precursors in the VZ.

Fgfr3 is also expressed transiently by a subpopulation of motoneurones (Philippe et al., 1998) and by late oligodendrocyte progenitors (late OLPs) just prior to terminal differentiation in vitro (Bansal et al., 1996). We are convinced that the *Fgfr3*$^+$ cells that we detect are not OLPs, however. First and foremost, double labelling for *Fgfr3* and *Pdgfra* (a marker of early OLPs) demonstrates that these mark separate populations of cells. The *Fgfr3*$^+$ and *Pdgfra*$^+$ cell populations appear at different times and initially their distributions are different. Moreover, the number and distribution of *Fgfr3*$^+$ cells was unaltered in neonatal *Pdgfa*-null spinal cords, which have very few *Pdgfra*$^+$ OLPs and oligodendrocytes (Fruttiger et al., 1999). This argues strongly that the large majority of *Fgfr3*$^+$cells revealed by our in situ hybridisation protocol are not OLPs. Bansal et al. (Bansal et al., 1996) have shown that OLPs do express *Fgfr3* mRNA in culture but only at a low level during the earlier stages of the lineage. Presumably this is below our limit of detection in situ. OLPs upregulate *Fgfr3* strongly just prior to oligodendrocyte differentiation (Bansal et al., 1996) but these presumably represent a small subset of OLPs in the embryonic spinal cord and do not feature in our analysis.

Fgfr3-positive cells co-expressed mRNA encoding glutamine synthetase (Gln; EC 6.3.1.2). In the CNS, Gln is an accepted marker of mature astrocytes (Norenberg and Martinez-Hernandez, 1979; Stanimirovic et al., 1999) but it is also expressed in oligodendrocytes (Domercq et al., 1999) and OLPS (Baas et al., 1998). Gln has not previously been ascribed to neuroepithelial precursors or immature astrocytes in the embryo, although *Gln* transcripts have been detected in the rat brain by northern blot as early as E14. To our knowledge, Gln has not been described in neurones except in pathological situations such as Alzheimer's disease (Robinson, 2000). Therefore, we are confident that the (*Fgfr3*$^+$, *Gln*$^+$) double-positive cells described here are glial cells. Taken together with the evidence against them being OLPs (see above), it seems likely that they correspond to immature and mature astrocytes. This is strongly supported by the observations that *Fgfr3*$^+$ cells co-express Gfap protein and/or mRNA in (1) the formative white matter of the normal developing spinal cord and (2) cultures of dissociated spinal cord cells.

Neuroepithelial origins of astrocytes

Fgfr3 was expressed in two domains of the spinal cord neuroepithelium separated by an *Fgfr3*-negative region. This was true of both rodent and avian embryos though it was more obvious in the latter. The *Fgfr3*-negative region corresponds roughly to the pMN domain of the VZ that generates somatic motoneurones followed by *Pdgfra*$^+$ OLPs (Sun et al., 1998; Rowitch et al., 2002). Therefore, our data indicate that OLPs and astrocytes originate from separate precursors that reside in different parts of the VZ. How does this fit with other ideas about the origin of astrocytes? One hypothesis is that astrocytes arise by transdifferentiation of radial glia, after the latter have fulfilled their role as cellular substrates for radial migration of neuronal progenitors (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). This could be compatible with our *Fgfr3* expression data, as radial glia have their cell bodies close to the ventricular

surface. However, radial glia are distributed all around the spinal cord lumen, unlike *Fgfr3*, so one would have to postulate that only a subset of radial glia express *Fgfr3*.

In double-knockout mice that lack the two basic helix-loop-helix (bHLH) transcriptions factors Olig1 and Olig2, the pMN domain of the VZ undergoes a homeotic transformation into p2, its immediate dorsal neighbour (Rowitch et al., 2002). As a result, pMN no longer generates motoneurones followed by OLPs, but instead produces V2 interneurones followed by astrocytes (Zhou and Anderson, 2002; Takebayashi et al., 2002). By implication, this is the usual fate of p2 precursors in wild-type mice. This is consistent with our observation that *Fgfr3*$^+$ astrocytes apparently originate within an extended part of the ventral VZ, including p2 but excluding pMN. Our *Fgfr3* expression data are also consistent with previous fate mapping experiments in chick-quail chimeras, which indicated that astrocytes are generated from dorsal as well as ventral parts of the VZ, whereas OLPs are generated only from ventral territory (Pringle et al., 1998). It remains to be seen whether astrocytes that are generated from distinct neuroepithelial domains (p3 or p2, say) have identical properties or whether they are functionally specialised – for modulating synaptic activity or interacting with blood vessels, for example.

Production of ventral cell types such as motoneurones, interneurones and OLPs is dependent on Shh signalling. As many *Fgfr3*-expressing astrocyte precursors appear to originate in p3, p2 and other ventral domains, we might expect that production of astrocytes might also depend on Shh signalling. However, we found that astrocytes developed in explant cultures of ventral neural tube either in the presence or absence of Shh activity. Our data imply that astrocytes are specified by different mechanisms than OLPs – at least, they demonstrate that astrocyte and OLP production are not obligatorily linked. In fact, there is evidence that more than one signalling pathway can lead to astrocyte development in vitro (Rajan and McKay, 1998). Because astrocytes can be formed from dorsal as well as ventral neuroepithelium, it remains possible that 'ventral' astrocytes might normally be under Shh control, but that by blocking Shh signalling we uncover an alternative 'dorsal' pathway for astrocyte development.

It has been reported that there are glial-restricted precursor cells (GRPs) in the embryonic rat spinal cord that are dedicated to the production of astrocytes and oligodendrocytes (Rao and Mayer-Proschel, 1997; Herrera et al., 2001). This seems to conflict with current evidence that oligodendrocytes and astrocytes are generated from different precursors in the embryonic spinal cord (Lu et al., 2002; Rowitch et al., 2002; Zhou and Anderson, 2002) (this paper). A possible reconciliation might be that GRPs with the potential to generate both astrocytes and oligodendrocytes are formed in all parts of the spinal cord VZ but are constrained in vivo to generate only astrocytes or only oligodendrocytes, depending on the signals in their local environment (i.e. where they are located) (for a review, see Rowitch et al., 2002).

Fgfr3 regulates Gfap expression in grey matter astrocytes

Astrocytes with distinct, heritable morphologies have been described in cultures of rat spinal cord cells (Fok-Seang and Miller, 1992). Astrocytes in different parts of the CNS differ in morphology or function in vivo too, suggesting that they might fulfil different, region-specific functions. In addition, astrocytes in white matter tracts generally have smaller cell bodies with more and longer processes compared to their counterparts in grey matter (Connor and Berkowitz, 1985). For this reason, white matter astrocytes are sometimes referred to as 'fibrous' and those in grey matter as 'protoplasmic' or 'velous'. White matter astrocytes also express high levels of Gfap, whereas grey matter astrocytes contain little or no immunoreactive Gfap.

Fibrous and protoplasmic astrocytes might develop from separate lineages (Connor and Berkowitz, 1985). However, our observation that Gfap is upregulated in grey matter astrocytes of *Fgfr3*-null mice provides strong in vivo evidence that extracellular signals might be required to maintain their normal Gfap-negative phenotype. This is consistent with a report that adding Fgf2 to cultured astrocytes downregulates Gfap mRNA and protein and causes their morphology to change (Reilly et al., 1998). Fgf2 and other known Fgfr3 ligands such as Fgf9 are made by, and presumably released from, many CNS neurones (Eckenstein et al., 1991; Cotman and Gomez-Pinilla, 1991; Woodward et al., 1992; Gomez-Pinilla et al., 1994; Kuzis et al., 1995). One possible reason that white matter astrocytes express high levels of Gfap in wild-type mice might be that they are denied exposure to *Fgfr3* ligands in axon tracts – perhaps because Fgf, like Pdgf, is secreted from neuronal cell bodies but not from axons (Fruttiger et al., 2000).

Upregulation of Gfap in the *Fgfr3*-null mouse is mindful of the astrocyte response to CNS injury or disease – so-called reactive gliosis or astrocytosis (for reviews, see Ridet et al., 1997; Norton, 1999). It would be interesting to know whether interruption of signalling through Fgfr3 is somehow involved in the astrocyte reaction to injury. However, it is unlikely to be straightforward, because Gfap upregulation in the *Fgfr3*-null animals does not occur until around 2 months of age, suggesting that it is an indirect effect. In addition, the data from the *Fgfr3*-null mouse are difficult to square with the observation that intra-ventricular injection of Fgf2 has been reported to increase the number of Gfap$^+$ reactive astrocytes (Unsicker, 1993).

Most grey matter (protoplasmic) astrocytes possess many short sheet-like processes containing little, if any, Gfap (Connor and Berkowitz, 1985). It has been suggested that this morphology might help them to infiltrate the neuropil and surround axonal terminals, synapses and neuronal cell bodies, consistent with one of their proposed roles in neurotransmitter metabolism (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). It will be interesting to see if the reactive astrocytes in *Fgfr3*-null mice are defective in neurotransmitter metabolism and whether this contributes to the premature death of the animals.

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The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal–Ventral Differences in GRP Cell Function

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We have found that the tripotential glial-restricted precursor (GRP) cell of the embryonic rat spinal cord can give rise *in vitro* to bipotential cells that express defining characteristics of oligodendrocyte-type-2 astrocyte progenitor cells (O2A/OPCs). Generation of O2A/OPCs is regulated by environmental signals and is promoted by platelet-derived growth factor (PDGF), thyroid hormone (TH) and astrocyte-conditioned medium. In contrast to multiple observations indicating that oligodendrocyte precursor cells in the embryonic day 14 (E14) spinal cord are ventrally restricted, GRP cells are already present in both the dorsal and ventral spinal cord at E13.5. Ventral-derived GRP cells, however, were more likely to generate O2A/OPCs and/or oligodendrocytes than were their dorsal counterparts when exposed to TH, PDGF, or even bone morphogenetic protein-4. The simplest explanation of our results is that oligodendrocyte generation occurs as a result of generation of GRP cells from totipotent neuroepithelial stem cells, of O2A/OPCs from GRP cells and, finally, of oligodendrocytes from O2A/OPCs. In this respect, the responsiveness of GRP cells to modulators of this process may represent a central control point in the initiation of this critical developmental sequence. Our findings provide an integration between the earliest known glial precursors and the well-studied O2A/OPCs while opening up new questions concerning the intricate spatial and temporal regulation of precursor cell differentiation in the CNS.

Key words: glial-restricted precursor cell; GRP cell; oligodendrocyte; O2A progenitor cell; OPCs; spinal cord development; ventral origin; neuroepithelial stem cells

Understanding how the differentiated cell types of the body are generated is a central challenge in developmental biology. Multiple components contribute to this process, including signaling molecules and transcription factors that cause precursor cells to progress along different developmental pathways. Central to understanding cell generation, however, is identification of the precursor cell from which a given cell type arises, for it is the specific precursor cell that represents the actual target for exogenous influences.

The creation of specific precursor cells and differentiated cell types proceeds through a sequence of lineage restrictions but also may involve a phenomenon of lineage convergence. Through lineage restriction, the totipotent stem cells of the earliest embryo generate progeny that are more restricted in the range of cell types they generate. For example, totipotent embryonic stem cells give rise to tissue-specific stem cells. Tissue-specific stem cells proceed to produce differentiated cell types via intermediate lineage-restricted precursor cells. These lineage-restricted precursor cells ultimately generate a subset of the differentiated cell types in a particular tissue. Lineage restriction is complemented in development by the process of lineage convergence, by which different lineages give rise to the same cell type. One example of such convergence is seen in the formation of cartilage from both mesenchymal and cranial neural crest lineage (Baroffio et al., 1991).

Studies on CNS development are revealing a rich diversity of precursor cells that can give rise to the same cell type, particularly with respect to glial development. For example, it is well established that oligodendrocytes can be generated from oligodendrocyte-type-2 astrocyte progenitor cells (Raff et al., 1983; Skoff and Knapp, 1991), which also are referred to as oligodendrocyte precursor cells (Raff et al., 1983; Skoff and Knapp, 1991) and abbreviated here as O2A/OPCs. More recent studies on embryonic rat spinal cord have led to the isolation of a new and distinct population, called tripotential glial-restricted precursor (GRP) cells, that also can generate oligodendrocytes *in vitro* and *in vivo* (Rao et al., 1998; Herrera et al., 2001). GRP cells and O2A/OPCs differ in several characteristics. For example, GRP cells and O2A/OPCs differ in their responses to mitogens, survival factors, and inducers of differentiation (Rao et al., 1998). GRP cells and O2A/OPCs also express distinct differentiation potentials *in vitro*: GRP cells are able to generate oligodendrocytes and two distinct astrocyte populations, whereas O2A/OPCs can generate oligodendrocytes and only one kind of astrocyte. Moreover, GRP cells readily generate astrocytes when transplanted into the neonatal or adult brain (Herrera et al., 2001), a cell type not generated from primary O2A/OPCs, after transplantation into the normal CNS (Espinosa de los Monteros et al., 1993).

Several critical questions arise from the fact that it now is possible to isolate two distinct precursor cell populations (i.e., GRP cells and O2A/OPCs) from the developing animal, each of

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Table 1. Differentiation potential of GRP-derived O4$^+$ and O4$^-$ cells

Culture condition	O4$^+$ cells		O4$^-$ cells	
	PDGF/TH	10% FCS	PDGF/TH	10% FCS
	Number of clones that contain specific cell types/number of all scored clones			
Type-2 astrocytes only	0/15	21/22	0/29	1/21
Type-1 astrocytes only	0/15	1/22	0/29	3/21
Type-1 and type-2 astrocytes	0/15	0/22	0/29	17/21
Oligodendrocytes and progenitors	15/15	0/22	29/29	0/21

Individual GRP cell-derived O4$^+$ and O4$^-$ cells were expanded to a clonal size of 5–10 cells before being exposed to 10% FCS to induce astrocytic differentiation or exposed to PDGF and TH to promote oligodendrocyte differentiation. After 5 or 10 d, respectively, clones were stained with anti-GFAP, A2B5, and anti-GalC antibodies. In serum-containing medium, all but one of the clones derived from O4$^+$ cells contained only astrocytes with the antigenic phenotype of type-2 astrocytes (i.e., GFAP$^+$ and A2B5$^+$). In contrast, clones generated from O4$^-$ cells contained a mixture of type-1 (i.e., GFAP$^+$A2B5$^-$) and type-2 astrocytes. Although some clones derived from O4$^+$ or O4$^-$ cells contained one or two progenitor cells, none of the clones contained oligodendrocytes at that time point. All clones exposed to PDGF and TH, regardless of being derived from O4$^+$ or O4$^-$ cells, contained A2B5$^+$ progenitor cells and GalC$^+$ oligodendrocytes. None of these clones contained astrocytes.

which can generate oligodendrocytes. Is the relationship between these two populations one of lineage restriction or lineage convergence? If GRP cells and O2A/OPCs are related, what signals promote the generation of one from the other and how can the existence of both populations be integrated with existing studies on the generation of oligodendrocytes during spinal cord development?

MATERIALS AND METHODS

Cell culture. A2B5$^+$ GRP cells were isolated from embryonic day 13.5 (E13.5) Sprague Dawley rat spinal cords by positive selection on immunopanning dishes coated with A2B5 antibody (Rao et al., 1998). GRP cells were then grown in the presence of 10 ng/ml basic FGF (bFGF) and indicated supplements for various time points on fibronectin/laminin-coated coverslips at 3000 cells/well for mass culture experiments or on coated grid dishes for clonal analysis. Cultures were fed every other day with the factors indicated. At the end of the experiment, cells were stained with O4 (Sommer and Schachner, 1981) or A2B5 antibodies to detect precursor cells, anti-galactocerebroside (GalC) antibody (Gard and Pfeiffer, 1990; Gard et al., 1995) to identify oligodendrocytes, and anti-GFAP antiserum to identify astrocytes (Norton and Farooq, 1993; Morita et al., 1997; Gomes et al., 1999) followed by appropriate fluorochrome-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL). The number of cells of each type relevant to each experiment was calculated, as was the total cell number. As originally defined, GFAP$^+$ cells were scored as type-2 astrocytes if they were stellate and A2B5$^+$ and as type-1 astrocytes if they were fibroblast-like in morphology and were A2B5$^-$.

Rationale for use of the O4 antibody in analyzing generation of O2A/OPCs from GRP cells. To determine whether one cell type gives rise to another, it is useful to identify a marker that is expressed by one cell type but not by the other. This is particularly problematic for analysis of GRP cells and O2A/OPCs. Freshly isolated GRP and O2A/OPCs both label with the A2B5 monoclonal antibody. We have shown previously that GRP cells can express receptors for platelet-derived growth factor (PDGF) without losing their tripotentiality (Rao et al., 1998). Our ongoing studies have revealed that tripotential GRP cells also label with anti-GD3 and anti-NG-2 antibodies (C. Pröschel, D. Gass, and M. Mayer-Pröschel, unpublished observations). Thus, none of these markers, which have been used by many others to study development of O2A/OPCs (Hart et al., 1989; Yim et al., 1995; Nishiyama et al., 1996), allow a distinction to be made between GRP cells and O2A/OPCs.

At this stage, the only remaining candidate marker for investigating whether GRP cells can generate O2A/OPCs is the O4 monoclonal antibody (Sommer and Schachner, 1981). This antibody can be used to define a secondary stage of O2A/OPC development, in which A2B5$^+$O4$^-$ O2A/OPCs give rise to cells that are A2B5$^+$ and also O4$^+$. The great majority of O2A/OPCs isolated from the p7 optic nerve are O4$^+$ (M. Noble, unpublished observations), whereas GRP cells are O4$^-$ (Rao and Mayer-Pröschel, 1997; Rao et al., 1998). In addition, it has been shown that development of GalC$^+$ oligodendrocytes in the O2A/OPC lineage is preceded by the appearance of cells that are O4$^+$ but GalC$^-$ (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). Critically, O4$^+$GalC$^-$ cells isolated from many regions of the postnatal CNS, including spinal cord, are bipotential cells capable of differentiating into both oligodendrocytes and type-2 astrocytes (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999). O4$^+$GalC$^-$ cells also can be induced to proliferate *in vitro* and in this respect are not terminally differentiated (Small et al., 1987; Trotter et al., 1989; Gard and Pfeiffer, 1990; Reynolds and Wilkin, 1991; Warrington and Pfeiffer, 1992; Avossa and Pfeiffer, 1993; Barnett et al., 1993; Gard et al., 1995). Thus, although some authors have preferred to consider O4$^+$GalC$^-$ cells (isolated from postnatal animals or derived from O2A/OPCs) as more committed "oligodendroblasts," the O4$^+$GalC$^-$ cells studied thus far express those characteristics (in particular, bipotentiality *in vitro* and ability to divide) that are most important in defining a cell as being a bipotential O2A/OPC.

Clonal analysis of E13.5 GRP cell-derived O4$^+$ cells. We confirmed the differentiation potential of a cell by clonal differentiation analysis, as used in our previous studies on GRP cells (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) and extensive studies on O2A/OPCs (Ibarrola et al., 1996; Smith et al., 2000); this is the only technique that allows the differentiation characteristics of individual precursor cells to be unambiguously ascertained. The basic strategy used to conduct such analyses in the present studies was as follows: GRP cells were isolated from E13.5 spinal cord as described previously and grown either for 24 hr or for 21 d in the presence of bFGF (10 ng/ml) before being exposed to the condition most effective at generating O4$^+$GalC$^-$ cells (i.e., chemically defined medium supplemented with 10 ng/ml PDGF–A chain homodimer; Peprotech, Rocky Hill, NJ) and thyroid hormone (TH; Sigma, St. Louis, MO). It is critical to note that GRP cells grown for 24 hr in FGF do not express PDGF receptor-α (PDGFR-α), whereas long-term cultured GRP cells express this receptor. We have determined that when grown in the presence of FGF, GRP cells remain tripotential regardless of their PDGF receptor status (Rao et al., 1998). After periods of additional *in vitro* growth indicated in Results, cultures were labeled with both O4 and anti-GalC antibodies, followed by appropriate fluorescein- and rhodamine-conjugated secondary antibodies. Fluorescence-activated cell sorting was then used to obtain populations of O4$^+$GalC$^-$ cells. O4$^+$GalC$^-$ cells were plated at clonal density and single O4$^+$GalC$^-$ cells were identified and circled. Cells were induced to divide for 5 d (in PDGF/bFGF at 10 ng/ml), and clones were switched to PDGF plus TH or 10% FCS when they reached a density of 5–10 cells. After 10 or 3 d, respectively, clones were stained with the A2B5, anti-GFAP, and anti-GalC antibodies. Control cells were switched to PDGF plus TH or 10% FCS without previous proliferation and stained after an additional 10 and 3 d, respectively. The results of our clonal analyses are shown in Tables 1 and 2.

Immunostaining of clones. Staining procedures were as described previously (Rao and Mayer-Pröschel, 1997). Briefly, the A2B5 and anti-GalC antibodies were grown as hybridoma supernatants (American Type Culture Collection, Manassas, VA) and used at a dilution of 1:2. The O4 hybridoma cell line was a generous gift from Ilse Sommer (University of Glasgow, Glasgow, UK), and its supernatant was also used at a 1:2 dilution. Anti-GFAP (polyclonal, rabbit anti-cow; purchased from Dako, Glostrup, Denmark) was used at a 1:100 dilution and applied overnight. All secondary antibodies [i.e., goat anti-mouse IgM-biotin, IgG3-tetramethylrhodamine B isothiocyanate, goat-anti-rabbit Ig (heavy and light chain)-FITC (Southern Biotechnology), and streptavidin (Molecular Probes, Eugene, OR)] were used at a 1:100 dilution. Anti-NG2

Table 2. NSC-derived A2B5$^+$ cells represent GRP cells

Antigenic phenotypes of cells within clones	Ventral E10.5 spinal cord-derived					Dorsal E10.5 spinal cord-derived				
	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^-$ only	A2B5$^+$ only	GalC$^+$	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^+$ only	A2B5$^+$ only	GalC$^+$
Percentage of clones (total number) after 7 d in 10% FCS/bFGF	70 (59)	0	0	30 (26)	0	83 (68)	0	0	17 (14)	0
Percentage of clones (total number) after 10 d in TH/bFGF	29 (33)	0	0	36 (40)	35 (39)	34 (23)	0	0	41 (29)	25 (18)

NSCs were isolated from dorsal and ventral regions of the E10.5 spinal cord and expanded in nondifferentiation conditions. After 3 d, dorsal and ventral cultures were allowed to differentiate, and the appearing A2B5$^+$ cells were harvested and replated at clonal density. Expanded clones were exposed either to 10% FCS or to TH to promote astrocytic and oligodendrocytic differentiation, respectively (both conditions also contained FGF). Both ventral- and dorsal-derived clones generated two types of astrocytes in the presence of 10% FCS and GalC$^+$ oligodendrocytes in the presence of TH. All clones that contained oligodendrocytes also contained A2B5$^+$ progenitor cells. We did not see any clones that contained only one type of astrocytes. The numbers shown refer to the number of clones containing different cell types but not the relative composition of the entire culture. For example, the great majority of astrocyte-containing clones in TH only had 1–5% GFAP$^+$ cells, suggesting that the presence of FGF allows some astrocyte differentiation to occur even when TH is present. Numbers in parentheses refer to the total number of clones, whereas numbers without parentheses indicate the percentage of clones in each category.

antiserum was a generous gift from Dr. W. Stallcup (Burnham Institute, La Jolla Cancer Research Center, CA) and was used at a 1:100 dilution.

RESULTS

Tripotential GRP cells, which are O4$^-$ cells, generate bipotential O4$^+$GalC$^-$ cells when grown in the presence of PDGF and thyroid hormone

The first question we addressed was whether tripotential GRP cells can generate cells with the antigenic and differentiation characteristics of bipotential O2A/OPCs. This question was investigated by a combined analysis of antigen expression and of differentiation potential at the clonal level. The requirement to use the O4 antibody (Sommer and Schachner, 1981) as a potential marker of O2A/OPCs is explained in Materials and Methods. Briefly, both GRP cells and O2A/OPCs label with the A2B5 antibody, the NG-2 antibody (Stallcup and Beasley, 1987), and the anti-GD3 antibody (Seyfried and Yu, 1985), and both populations can express PDGF receptors while maintaining their characteristic differentiation potential. Thus, of all of the markers that have been used to study the ancestors of oligodendrocytes, it was only the O4 antibody that remained potentially useful in this context. We designed experiments that would allow us to answer the following questions: (1) are there *in vitro* growth conditions that promote the generation of O4$^+$GalC$^-$ cells from O4$^-$ GRP cells, and (2) do GRP cell-derived O4$^+$GalC$^-$ cells still behave like tripotential GRP cells or do they now behave like bipotential O2A/OPCs?

We first examined the effects on GRP cells of a wide variety of conditions (see Materials and Methods) shown previously to induce generation of oligodendrocytes in cultures of O2A/OPCs. Although astrocyte-conditioned medium in combination with TH was the most effective condition for inducing the appearance of oligodendrocytes over a 3 d time period (data not shown), it was growth in the presence of FGF plus PDGF plus TH that was associated with the generation of the greatest proportion of O4$^+$GalC$^-$ cells.

In cultures of freshly isolated GRP cells that were grown for 24 hr in the presence of FGF and then additionally exposed to PDGF plus TH (with FGF still present), 78 ± 9% of the cells were O4$^+$GalC$^-$ after 3 d in culture. In addition, we noticed that 20 ± 5% of all cells were O4$^+$GalC$^+$ oligodendrocytes and a small percentage (2 ± 0.7%) of cells represented GFAP$^+$ astrocytes. We never observed the appearance of any cells that were GalC$^+$ but O4$^-$, consistent with previous observations that passage through an O4$^+$ stage is required before the expression of GalC immunoreactivity (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). GRP cell cultures that were grown in the presence of FGF alone contained no O4$^+$ cells, and previous studies have demonstrated that GRP cells expanded in this manner retain the ability to generate oligodendrocytes, type-1 astrocytes, and type-2 astrocytes.

Although previous studies have shown that O4$^+$GalC$^-$ cells isolated from postnatal animals or derived from bipotential O2A/OPCs are bipotential *in vitro* (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999), it cannot be assumed that such differentiation characteristics necessarily apply to O4$^+$GalC$^-$ cells derived from tripotential GRP cells. To determine the differentiation potential of GRP cell-derived O4$^+$GalC$^-$ cells, we cultured expanded GRP cells in the presence of FGF for several days, grew them in the additional presence of PDGF plus TH for 3 more days, purified the O4$^+$GalC$^-$ cells, and analyzed their differentiation potential in clonal cultures. Extending the previous expansion period in FGF in this manner resulted in a higher percentage of the cells in the culture remaining O4$^-$, thus allowing the study of this population also.

Cloned O4$^+$GalC$^-$ cells derived from GRP cells expressed the bipotential differentiation characteristics associated with O2A/OPCs. When grown in conditions that induced generation of astrocytes, O4$^+$GalC$^-$ cells derived from GRP cells exhibited the typical differentiation response of O2A/OPCs. In the presence of 10% FCS, the only astrocytes generated in 21 of 22 clones derived from O4$^+$GalC$^-$ cells were type-2 astrocytes (i.e., A2B5$^+$GFAP$^+$ stellate cells; Table 1 and Fig. 1*A*). Only one clone generated type-1-like astrocytes (i.e., A2B5$^-$GFAP$^+$ cells with a fibroblast-like morphology), a frequency low enough to be consistent with the possibility that this one clone had been mislabeled at the beginning of the experiment. This outcome was very different from that obtained with GRP cells themselves, clones of which generate a combination of type-1 and type-2 astrocytes in these conditions (Rao et al., 1998). Moreover, the O4$^-$GalC$^-$ cells that remained after the purification process were still tripotential, emphasizing that the acquisition of bipo-

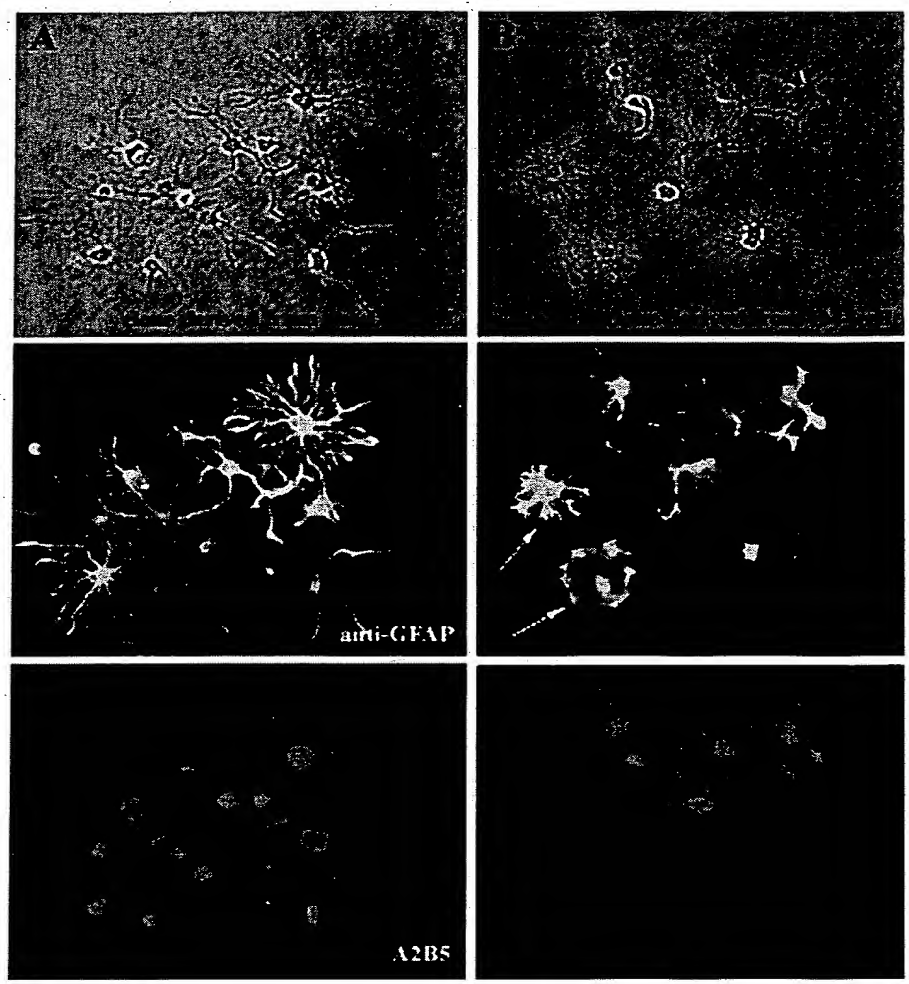

Figure 1. GRP-derived O4$^+$ cells are bipotential and represent O2A/OPC-like cells. Freshly isolated GRP cells were grown for 3 weeks in defined medium in the presence of bFGF and then switched to a medium supplemented with bFGF and TH; after 5 d, cultures were stained with the O4 antibody (see Materials and Methods). Cells were then dislodged from the surface and plated at clonal density in poly-L-lysine-coated dishes. Single O4$^+$ cells were circled. After 3 d in culture, cells were exposed to medium supplemented with bFGF and 10% FCS. (Parallel experiments using BMP4 instead of FCS yielded identical results.) After 5 d, clones were stained with A2B5 (rhodamine), anti-GFAP (fluorescein), and anti-GalC (coumarin) antibodies. The coumarin staining is not shown because none of the clones contained any GalC$^+$ oligodendrocytes in this condition. *A*, Clone derived from a single O4$^+$GalC$^-$ cell. The progeny from O4$^+$ founder cells consists exclusively of A2B5/GFAP double-positive cells, consistent with the antigenic phenotype of type-2 astrocytes. *B*, Clone derived from a single O4$^-$GalC$^-$ cell. The progeny from O4$^-$ founder cells consists of A2B5/GFAP double-positive type-2 astrocytes and A2B5$^-$GFAP$^+$ cells, representing type-1 astrocytes (indicated by *arrows*).

tentiality was a specific event and not merely associated with aging, even in conditions that promote the transition to a bipotential phenotype. When grown in the presence of 10% FCS, O4$^-$GalC$^-$ cells generated clones containing a mixture of type-1 and type-2 astrocytes (Table 1 and Fig. 1*B*), and thus behaved as GRP cells. In contrast to this difference with respect to astrocyte induction, both O4$^+$GalC$^-$ and O4$^-$GalC$^-$ cell-derived clones contained oligodendrocytes when grown in the presence of PDGF plus TH.

The simplest explanation of the data obtained in the above experiments is that GRP cells can generate O4$^+$GalC$^-$ cells that exhibit *in vitro* the defining bipotential differentiation restriction of O2A/OPCs. The results also indicate that generation of such bipotential cells is an environmentally regulated differentiation event, for which PDGF and TH represent potent inducing agents.

GRP cells can be isolated from both ventral and dorsal E13.5 spinal cord

A critical component of the current understanding of oligodendrocyte development *in vivo* is that specific precursor cells for oligodendrocytes first appear in the ventral spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). We subsequently determined whether GRP cells are selectively localized in the ventral spinal cord at or before the time when putative oligodendrocyte precursor cells first appear ventrally. Because previous studies have shown that GRP cells are already present at E13.5 (Rao et al., 1998), we microdissected dorsal and ventral portions of the E13.5 cord to determine the regional distribution of GRP cells; this is a half day earlier than the earliest reported appearance of specific oligodendrocyte precursor cells, as defined by expression of the PDGF receptor (Hall et al., 1996). Freshly isolated cells from dorsal and ventral cord were immunolabeled with A2B5 antibody, purified by fluorescence-activated cell sorting, and plated at clonal density on grid dishes in different conditions as described below. In three independent experiments, the dorsal spinal cord consistently contained an average of 19 ± 8% A2B5$^+$ cells, whereas the ventral portion contained an average of 52 ± 7% A2B5$^+$ cells. Thus, although the ventral cord contained a higher proportion of A2B5$^+$ cells than did the dorsal cord, such cells were found in both regions of the cord.

To determine whether dorsal- and ventral-derived A2B5$^+$ cells

were GRP cells, the A2B5$^+$ clones were first grown in the presence of bFGF until they reached a size of 10–20 cells. Astrocytic differentiation was then induced by exposing cultures for 3 d to 10% FCS. All clones contained both A2B5$^-$GFAP$^+$ type-1 astrocytes and A2B5$^+$GFAP$^+$ type-2 astrocytes independent of their site of isolation. Thus, these cells were typical of GRP cells in their ability to generate two distinct astrocyte populations. Generation of oligodendrocytes was also possible with both ventral- and dorsal-derived cells, as discussed in the following section.

GRP cells derived from both the ventral and dorsal E13.5 spinal cord can generate O2A/OPCs, oligodendrocytes, and astrocytes

Because expression of PDGF receptor-α in the E14 spinal cord has been interpreted to be an indication of a preferential ventral origin of oligodendrocytes (Pringle and Richardson, 1993; Hall et al., 1996), we asked whether ventral- and dorsal-derived GRP cells differed in their ability to generate O2A/OPCs and/or oligodendrocytes. GRP cells were isolated from ventral or dorsal E13.5 spinal cord as described in the preceding section. Freshly isolated cells were plated at a low density on coverslips in the presence of FGF and exposed to conditions (PDGF plus TH) that would induce the transition into O2A/OPCs (as determined previously) or to conditions that would potentially inhibit a transition into O2A/OPCs. As a potential inhibitor molecule, we used bone morphogenetic protein-4 (BMP4), which has been shown to inhibit oligodendrocyte generation (Mabie et al., 1997; Grinspan et al., 2000; Mehler et al., 2000; Zhu et al., 2000) and is present in the embryonic neural tube (D'Alessandro and Wang, 1994; Barth et al., 1999; Grinspan et al., 2000; Liem et al., 2000). After 3 d of *in vitro* growth in the condition discussed, cells were stained with the O4 monoclonal antibody and with anti-GalC and anti-GFAP antibodies.

In the presence of PDGF and TH, cells from both the dorsal and ventral spinal cord were able to generate O4$^+$GalC$^-$ cells with equal frequencies but differed with respect to oligodendrocyte generation (Fig. 2). Specifically, 88 ± 6% of dorsal-derived cells were O4$^+$GalC$^-$, 3 ± 2% were GalC$^+$ oligodendrocytes, and 3 ± 2% were GFAP$^+$ astrocytes. In contrast, 74 ± 9% of ventral-derived cells were O4$^+$GalC$^-$, 28 ± 5% were GalC$^+$ oligodendrocytes, and 2 ± 1% were GFAP$^+$ astrocytes. Thus, although both dorsal and ventral cells were able to generate O2A/OPCs, only ventral-derived cells generated a significant number of GalC$^+$ oligodendrocytes over a 5 d time period. The lack of oligodendrocytes in dorsal cultures is not likely to be attributable to preferential cell death. because the total number of cells was not different in dorsal and ventral cultures (327 ± 8 and 326 ± 38. respectively). In addition, dorsal-derived cells demonstrated an equal ability to eventually generate oligodendrocytes. If cultures were examined after 10 d in the presence of TH, instead of after 5 d, then 69 ± 15% of the ventral cells and 73 ± 3% of dorsal cells were oligodendrocytes (data not shown).

Differences between dorsal- and ventral-derived GRP cells were also observed in response to BMP4. When dorsal- or ventral-derived GRP cells were grown in the presence of BMP4 in concentrations ranging from 1 to 100 ng/ml over 3 d, we observed that BMP4 promoted the generation of astrocytes in both dorsal and ventral cells (Fig. 3). At a low BMP concentration (1 ng/ml), ventral cells were more likely to differentiate into astrocytes than were dorsal cells (45 ± 4% vs 14 ± 4%, respectively). The preferential generation of GFAP$^+$ cells in ventral

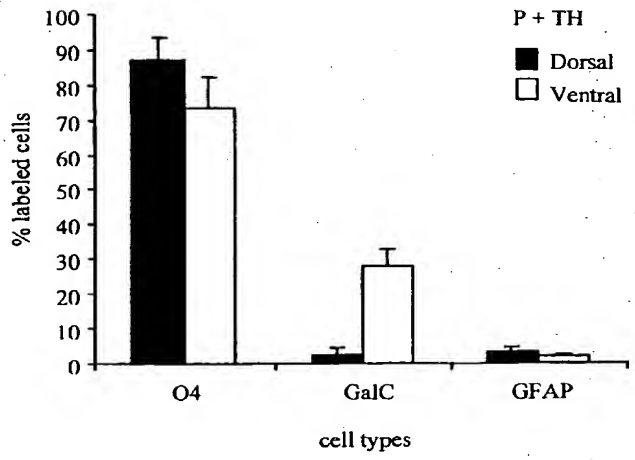

Figure 2. Both dorsal- and ventral-derived GRP cells generate O4$^+$GalC$^-$ cells. A2B5$^+$ cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated in the presence of bFGF supplemented with PDGF plus TH (*P + TH*) for 7 d. Dorsal and ventral cultures were then stained with O4, anti-GalC, and anti-GFAP antibodies. Both dorsal- and ventral-derived cultures generated comparable numbers of O4$^+$ precursor cells. However, GalC$^+$ oligodendrocytes were found predominantly in ventral-derived GRP cell cultures. Only a small fraction of both dorsal and ventral cultures gave rise to GFAP$^+$ astrocytes. Two independent experiments examining six data points for each condition revealed comparable results.

cultures was a transient phenomenon, in that only in dorsal-derived cultures did these numbers increase over the next several days (as discussed in the following paragraph). The addition of 10 ng/ml BMP had an identical effect on ventral and dorsal cells (48 ± 3% and 54 ± 7% astrocytes, respectively). The most dramatic difference between ventral and dorsal cells was observed at high BMP doses (100 ng/ml). In this condition, ventral cells responded with cell death rather than cell differentiation. In contrast, dorsal-derived GRP cells differentiated almost completely into astrocytes when exposed to 100 ng/ml BMP4.

Because BMP4 at 1 ng/ml revealed differences between dorsal- and ventral-derived GRP cells in the absence of toxicity, we subsequently examined the generation of O4$^+$GalC$^-$ cells in this culture condition (Fig. 4*A*). Cells were plated at a low density on coverslips in the presence of FGF and BMP4 (1 ng/ml) and examined after 7 d to allow for the generation of O4$^+$GalC$^-$ cells and/or GalC$^+$ oligodendrocytes. In cultures of GRP cells derived from dorsal spinal cord, the majority of cells (87 ± 8%) differentiated into GFAP$^+$ astrocytes, and only 12 ± 7% of the cells were O4$^+$GalC$^-$. We did not observe any GalC$^+$ oligodendrocytes in these cultures. In contrast, when ventral-derived cells were exposed to 1 ng/ml BMP for 7 d, 47 ± 8% differentiated into astrocytes (as observed for 3 d time point discussed previously) and 52 ± 7% of the cultures consisted of O4$^+$GalC$^-$ cells. Again, we did not observe any GalC$^+$ oligodendrocytes. Thus, BMP4 exposure was associated with a strikingly more significant decrease in the number of O4$^+$GalC$^-$ cells in dorsal- than in ventral-derived GRP cells.

We subsequently determined whether the addition of TH, a potent inducer of the generation of O4$^+$GalC$^-$ cells and/or oligodendrocytes, could counteract the effects of BMP4 (Fig. 4*B*). Dorsal and ventral cells were exposed to BMP at 1 ng/ml in the presence of TH at 50 nM for 7 d before the cultures were labeled

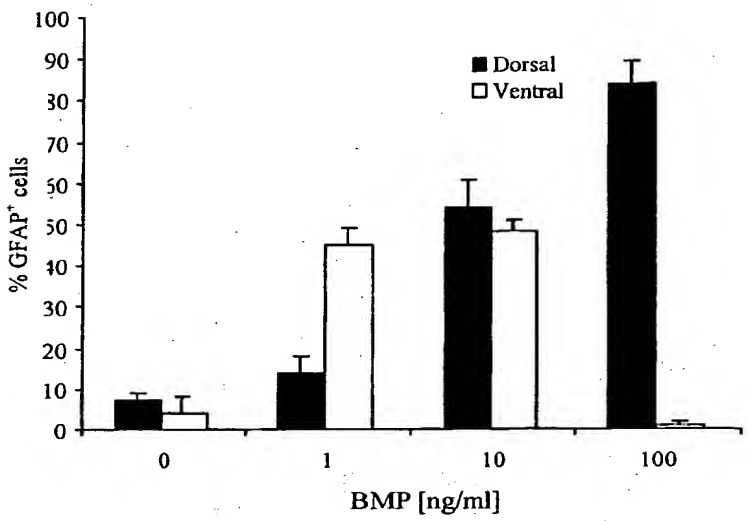

Figure 3. BMP4 induces differentiation of astrocytes from dorsal- and ventral-derived A2B5$^+$ cells in a dose-dependent manner. A2B5$^+$ cells, isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos, were plated at a low density in the presence of bFGF and increasing concentrations of BMP4 (0.1–100 ng/ml). After 3 d, cultures were labeled with anti-GFAP antibodies and the number of astrocytes was determined. Whereas dorsal cultures exhibited a continuous, dose-dependent increase in the number of GFAP$^+$ astrocytes, ventral-derived GRP cells generated significantly more astrocytes at lower doses of BMP (1 ng/ml) at this time point, and higher doses of BMP4 (100 ng/ml) proved to be lethal to ventral-derived GRP cells.

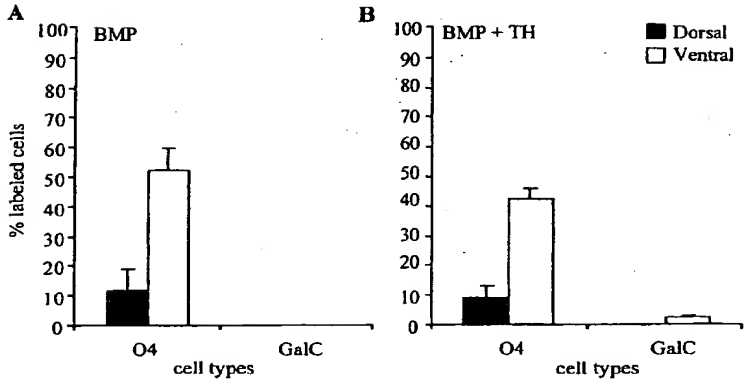

Figure 4. Differential effects of BMP4 on dorsal- and ventral-derived GRP cells. GRP cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated at a low density in the presence of FGF and BMP4 (*A*) (1 ng/ml) or FGF, BMP4 (1 ng/ml), and TH (*B*). To allow for oligodendrocyte generation, cultures were examined after 7 d for the presence of O4$^+$GalC$^-$ precursor cells or GalC$^+$ oligodendrocytes. Although GalC$^+$ oligodendrocytes were only found in ventral GRP cell cultures containing TH, both dorsal- and ventral-derived cultures contained O4$^+$GalC$^-$ precursor cells. In the presence of BMP, the ability of dorsal GRP cells to generate O4$^+$GalC$^-$ precursor cells was lower than that of ventral-derived cultures; this was not changed by the addition of TH.

with O4, anti-GalC, and anti-GFAP antibodies. As shown in Figure 4*B*, the addition of TH had little or no effect on the generation of O4$^+$GalC$^-$ cells in both dorsal and ventral cultures. However, we did detect a small but significant increase ($p < 0.002$) in the number of GalC$^+$ oligodendrocytes specifically in the ventral-derived cells. This effect was not seen in dorsal-derived cultures.

GRP cells can be generated from dorsal and ventral neuroepithelial stem cells of the E10.5 spinal cord

Our results thus far demonstrate that there is a dorsal–ventral gradient in GRP cell distribution in the spinal cord of the E13.5 rat, and that dorsal- and ventral-derived GRP cells are dissimilar in their abilities to generate oligodendrocytes over short time periods *in vitro*. In addition, these two populations differ in their response to BMP. Because GRP cells themselves are derived from neuroepithelial stem cells (NSCs) (Rao and Mayer-Pröschel, 1997), we subsequently determined whether dorsal- and ventral-derived NSCs differed in their capacity to generate GRP cells.

In these experiments, E10.5 spinal cord [at which time point all cells are NSCs (Kalyani et al., 1997)] was microdissected into dorsal and ventral regions. Dissociated cells were plated at clonal density on fibronectin/laminin-coated grid dishes in the presence of 10 ng/ml bFGF and embryonic chick extract (CEE), a condition that prevents differentiation of NSCs (Kalyani et al., 1997). After 3 d in culture, when clones reached a size of 20–50 cells, CEE was removed to allow the clones to differentiate into lineage-restricted precursor cells (Kalyani et al., 1997; Mayer-Pröschel et al., 1997; Rao and Mayer-Pröschel, 1997). After 5 d in the absence of CEE, clones were stained with A2B5 antibody and the number of clones containing A2B5$^+$ cells was determined.

Both dorsal- and ventral-derived NSCs generated A2B5$^+$ cells with a similar efficiency. From a total number of 175 ventral-derived NSC clones, 152 (i.e., 87%) contained A2B5$^+$ cells after 5 d of *in vitro* growth. Similarly, 200 of 213 (84%) dorsal-derived NSC clones cell clones contained A2B5$^+$ cells at this time point. Analysis of the differentiation potential of A2B5$^+$ cells derived from dorsal and ventral NSCs confirmed that these cells expressed the differentiation characteristics of GRP cells (Table 2). These experiments were performed as described previously (Rao and Mayer-Pröschel, 1997). Briefly, clones were stained with A2B5 as live cells and single clones were picked and replated into grid dishes. Single A2B5$^+$ cells were marked and expanded in the presence of bFGF. After clones reached a size of 20–40 cells (5 d), they were switched to 10% FCS to generate astrocytes. After 7 d, clones were stained with A2B5 and anti-

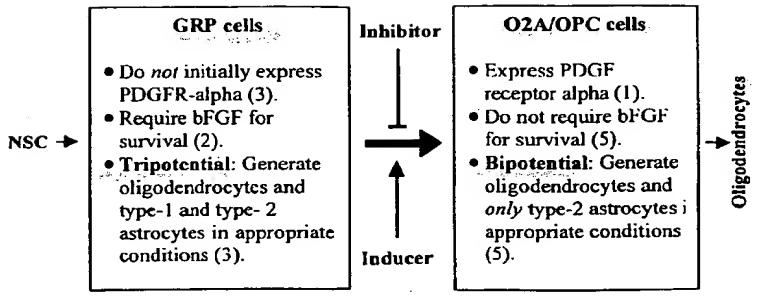

Figure 5. Sequential lineage restriction in the glial lineage of the CNS. A side-by-side comparison of the salient features of two lineage-restricted glial precursors of the CNS is shown. The evidence presented here strongly suggests a progressive and sequential transition from the tripotential GRP cell to the bipotential O2A/OPC. In the developing spinal cord, it currently seems most likely that this transition is controlled in a temporal and spatial pattern and is regulated by cell-extrinsic signaling molecules (Pringle et al., 1992; Rao and Mayer-Pröschel, 1997; Rao et al., 1998).

GFAP antibodies. All astrocyte-containing clones always contained a mixture of type-1 and type-2 astrocytes, regardless of whether they were generated in dorsal- or ventral-derived cultures and whether they were generated in response to FCS (or BMP, data not shown). A smaller number of clones consisted of $A2B5^+$ cells only, and none of these clones contained oligodendrocytes. (Table 2). In contrast, exposure of clones to PDGF plus TH for 10 d was associated with oligodendrocyte generation in all clones. Although differences were not striking, significantly more ventral clones generated oligodendrocytes than did dorsal clones over this time period (35 ± 7% vs 25 ± 2%, respectively; $p < 0.02$).

DISCUSSION

One of the essential challenges that arises with the discovery of any new precursor cell population is to determine how these cells might be integrated into (or might alter) existing views on tissue development. In the present studies on the tripotential GRP cell of the embryonic rat spinal cord, we have found that this recently discovered novel glial precursor cell can generate progeny with the antigenic phenotype and differentiation characteristics of bipotential O2A/OPCs. This process is regulated by cell-exogenous signaling molecules, with growth in the presence of PDGF plus TH being particularly effective in promoting such differentiation. In contrast to previous suggestions that putative oligodendrocyte precursor cells are localized in ventral regions of the E14 spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000), GRP cells could be isolated from both the dorsal and ventral cord of E13.5 rats. However, there were differences between dorsal- and ventral-derived GRP cells in their response to conditions that promote or inhibit generation of O2A/OPCs or oligodendrocytes, with ventral-derived GRPs exhibiting a greater propensity to differentiate along the oligodendrocyte lineage.

The demonstration that GRP cells can yield O2A/OPCs integrates these two glial precursor cell populations for the first time and indicates that their relationship is one of sequential lineage restriction rather than being independent precursors that generate oligodendrocytes. In light of our present studies, the simplest model of oligodendrocyte generation that appears to be consistent with all available data would be that production of these cell types requires the initial generation of GRP cells from NSCs followed by the generation of O2A/OPCs from GRP cells (Fig. 5). Our previous studies (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) indicated strongly that GRP cells are a necessary intermediate between NSCs and differentiated glia, and our present studies raise the possibility that O2A/OPCs are a necessary intermediate between GRP cells and oligodendrocytes. Despite the fact that both GRP cells and O2A/OPCs are $A2B5^+$, it seems unlikely that the $O4^+GalC^-$ cells studied in our *in vitro* experiments were derived from a subset of $A2B5^+O4^-$ bipotential O2A/OPCs present in the original GRP cell culture. In our previous characterizations of GRP cells derived from E13.5 spinal cords, we consistently failed to find clones that gave rise exclusively to type-2 astrocytes when exposed to 10% FCS, even when cells were serially recloned three times over a period of several weeks (Rao et al., 1998). Moreover, analysis of hundreds of putative GRP cell clones thus far has failed to reveal clones that generate only type-2 astrocytes when exposed to FCS or BMPs (Mayer-Pröschel, unpublished observations). Thus, it appears that the generation of cells with the characteristics of O2A/OPCs is a differentiation event that requires exposure of GRP cells to appropriate inductive signals, such as PDGF plus TH. Moreover, we could find no $GalC^+O4^-$ oligodendrocytes in any conditions, which would have at least raised the possibility that oligodendrocytes might be generated directly from GRP cells. Such results are consistent with previous observations that passage through an $O4^+GalC^-$ stage of development is required for oligodendrocyte generation from bipotential O2A/OPCs (Gard and Pfeiffer, 1990, 1993; Gard et al., 1995). Our data are also consistent with other studies indicating that $O4^+GalC^-$ cells are bipotential (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999).

It remains formally possible that GRP cells might be able to generate oligodendrocytes without passage through an intermediate O2A/OPC stage, or that NSCs could generate O2A/OPCs without going through a GRP cell stage. Nonetheless, it is important to stress that no data exist to support the possibility that O2A/OPCs are directly generated from NSCs or that oligodendrocytes are directly generated from either NSCs or GRP cells. Thus, the developmental pathway we suggest is at present the only one supported by experimental observations.

It is of particular interest to find that ventral-derived GRPs seem to differ from dorsal cells in such a manner so as to have an increased probability to generate O2A/OPCs and/or oligodendrocytes, even in the presence of BMP. Thus, it may prove necessary not only to study GRP cells but also to focus attention on ventral-derived GRP cells to understand the mechanism of action of those factors that eventually lead to oligodendrocyte generation. It will be of considerable interest to determine whether these differences are intrinsic to ventral- or dorsal-derived GRP cells or are acquired as a consequence of exposure to particular environmental signals. It also will be of interest to determine whether the O2A/OPCs generated from dorsal and ventral GRP cells themselves differ in their responsiveness to

inducers of oligodendrocyte generation, an interpretation that would be consistent with our data (Fig. 4*B*). In addition, our observation that the responsiveness of GRP cells to PDGF plus TH as promoting signals of O2A/OPC and oligodendrocyte generation may decrease with increased GRP cell expansion *in vitro* is reminiscent of our previous findings that O2A/OPCs expanded for continued periods become less responsive to PDGF as a mitogen (Bogler et al., 1990). Although the biological implications of this observation with respect to GRP cell biology require additional investigation, this result does emphasize the importance of expanding precursor cell populations *in vitro* as minimally as possible in studies on the function of exogenous signaling molecules.

It is important to consider the question of whether all previous studies attempting to define the early origin of the oligodendrocyte lineage have in fact been describing early differentiation events affecting GRP cells. It is clear from our previous work that GRP cells can express the PDGFR without losing their tripotential character (Rao et al., 1998). In addition, our ongoing work (Pröschel, Gass, and Mayer-Pröschel et al., unpublished observations) is demonstrating that GRP cells can also be NG-2$^+$ and GD3$^+$, two other antigens that have been used in studies on O2A/OPCs (Mayer-Pröschel, unpublished observations). Moreover, it currently appears that GRP cells are the dominant (if not exclusive) A2B5$^+$ cell population in the spinal cord until as late as E17 (Mayer-Pröschel, unpublished observations). Thus, it is beginning to seem likely that events such as expression of PDGFR in ventral A2B5$^+$ cells may reflect a differentiation process in GRP cells rather than the transition to being an O2A/OPC. Analyzing the early stages of generation of O2A/OPCs from GRP cells, whether *in vitro* or *in vivo*, will require identification of a marker that can be used to antigenically distinguish GRP cells from the A2B5$^+$O4$^-$ stage of O2A/OPCs. As indicated, none of the markers currently available seem to enable this distinction.

The field of developmental neurobiology is in the early stages of determining the relationship between different lineage-restricted precursor cells in the CNS, and our present experiments represent a critical step in determining whether GRP cells may be the ancestors of all glial populations of the spinal cord. Our present observations are consistent in two ways with such a suggestion. First, if this hypothesis were to be correct, then GRP cells should be able to give rise to O2A/OPCs (as we have found). We also would anticipate that GRP cells would be found in both the dorsal and ventral cord, although they may generate different progeny in these two regions. In future studies, it will be important to discover whether precursor cells with the properties of GRP cells also exist in other regions of the CNS. In addition, it will be important to determine whether other progeny of GRP cells include the A2B5$^+$ astrocyte precursor cells present in embryonic (E17) spinal cord and originally described by Fok-Seang and Miller (1992, 1994), the putative astrocyte precursor cells from the embryonic mouse cerebellum described by Seidman et al. (1997), the astrocyte precursor cells described by Mi and Barres (1999), or the pre-O2A progenitor cell described by Grinspan et al. (1990). In addition, it is of importance to determine whether the developmental inter-relationships that seem to exist in the spinal cord also apply to development of the brain. By identifying the relationship between these developmental pathways and the signals responsible for these transitions, we will move closer to a comprehensive understanding of glial development in the CNS.

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PATENT
Docket No.: 19603/1426 (CRF D-2238A)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Richard Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	To Be Assigned	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

DECLARATION OF STEVEN A. GOLDMAN UNDER 37 C.F.R. §1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor of Neurology and Neuroscience at Weill Medical College at Cornell University and an Attending Neurologist at New York Presbyterian Hospital.

3. I am a named inventor of the above patent application.

Kirschenbaum Article

4. I am a co-author of Kirschenbaum, et. al., "*In Vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain," Cerebral Cortex 6: 576-89 (1994) ("Kirschenbaum").

5. The study described in Kirschenbaum was carried out in my laboratory and I was the senior, supervising scientist on the study; therefore, I fully understand this work. Kirschenbaum cultures samples of adult temporal lobes under conditions suitable for neuronal differentiation, while exposed to ^{3}H-thymidine. These samples were incubated for 7-28 days, stained for neuronal and glial antigens, and autoradiographed. Neuron-like cells were found in explant outgrowths and monolayer dissociates of the subependymal zone and periventricular white matter but not the cortex. A small number of Map-2$^+$ and Map-5$^+$/glial fibrillary acidic protein$^-$ cells did incorporate ^{3}H-thymidine, suggesting neuronal production from precursor mitosis. However, the O4$^+$ oligodendrocytes were postmitotic. Even though the abstract of Kirschenbaum states that "O4$^+$ oligodendrocytes, although the predominant cell type, were *largely* postmitotic (emphasis in original)", I said this only because I am generally reluctant to make conclusions in absolute terms. Nevertheless, it is clear from the following statement on page 582 of Kirschenbaum that, in fact, *all* of the oligodendrocytes were post-mitotic:

> These O4$^+$/GFAP$^\pm$ cells were mitotically quiescent; among a sample of 8044 such cells, culled from four plates of subcortical white matter (2011 ± 858.6 O4$^+$ cells/plate, mean ± SD), *none* incorporated ^{3}H-thymidine *in vitro*, despite the frequent observation of ^{3}H-thymidine-labeled astrocytes in the same plates (emphasis in original).

The failure of the Kirschenbaum study to identify mitotic oligodendrocyte progenitor cells caused me to continue working to identify and produce such cells. These efforts were ultimately successful in producing the invention of the present application.

Bottenstein Patent

6. U.S. Patent No. 5,276,145 to Bottenstein ("Bottenstein") is directed to substantially purified preparations containing a neural progenitor regulatory factor that is important in regulating and coordinating production of oligodendrocytes and type 2 astrocytes. The identification of this factor was carried out with brain cells derived from neonatal rats of 1-3 days of age. These cells represented a mixture of cell types, that included "progenitors", "Type 2 Astrocytes", "Early Oligodendrocytes", "Late Oligodendrocytes", "Total Oligodendrocytes", "Type 1 Astrocytes", and "Microglia".

7. There are fundamental differences between the biology of rat and human oligodendrocyte progenitor cells. These are unaddressed in Bottenstein, which discusses findings

limited to neonatal rat brain. Whereas rat oligodendrocytes appear to retain mitotic potential, human oligodendrocytes do not (see Kirschenbaum). As a result, the oligodendrocyte progenitor cell of the rat brain cannot be considered homologous to its human counterpart. In particular, methods that permit the selective extraction and/or growth of oligodendrocyte progenitors from the rat brain do not differentiate between oligodendrocyte progenitor cells and mature oligodendrocytes able to re-enter the mitotic cycle. In humans, these constitute two discrete phenotypes, lineally related but temporally distinct. Our present invention teaches the selective acquisition of a highly enriched – to virtual purity - mitotically-competent oligodendrocyte progenitor cell pool, operationally separate and distinct from post-mitotic or mature oligodendrocytes.

8. Bottenstein was directed at the enrichment of glial progenitor cells from newborn rat brain. Newborns have an abundant population of still-developing oligodendrocyte progenitor cells that may constitute a significant fraction of all of the cells in neonatal brain tissue. Bottenstein reported that >30% of the cells of its tissue dissociates expressed the marker of this phenotype. With the addition of B104 conditioned media and the neural progenitor regulatory factor, this fraction increased to just over 40%. The nature of these cells is that of a still-mixed pool, in that the following populations appear to be represented by Bottenstein's data: astrocytes, oligodendrocytes, and a mixture of oligodendrodendroglial lineage cells of widely different developmental stages.

9. In contrast to the cells acquired from newborn rats using the Bottenstein protocol, the present invention is achieved with a procedure that permits, in both young and old humans, the selective extraction of progenitor cells strongly biased to oligodendrocytic phenotype, and allows the purification of these cells, including those from tissues in which they are scarce (e.g., postnatal and adult brain tissues harboring <1% of the desired oligodendrocyte progenitor cell type). In Example 5 of the present patent application, we reported the virtual purification of oligodendrocyte progenitor cells from tissues with a P/CNP2 promoter-targeted FACS-defined incidence of <1%. This constituted a far greater enrichment of the oligodendrocyte progenitor cell (i.e. 170-fold) than that achieved by Bottenstein (i.e. less than 1.5-fold) and yields a far more pure product of oligodendrocyte progenitor cells.

10. In contrast to Bottenstein, the human oligodendrocyte progenitor cell populations achieved through our protocols are virtually pure as to phenotype. Compare Figure 5B to its control, Figure 5A. In Figure 5A, the gated single cell represents the false-positive sort incidence. Such incidences constitute <1% of the frequency of events noted in Figure 5B, indicating >99% purity of the P/CNP2:hGFP-sorted oligodendrocyte progenitor cells. This can be modulated as a function of sort speed to achieve any desired degree of purity, the trade-off being lower yields as higher degrees

of purification are achieved. By virtue of the high-purity extraction attainable by fluorescence-activated cell sorting, the progenitor cells we produce are never exposed to paracrine factors released by other cells, after removal from tissue. This permits their maintenance in an undifferentiated and phenotypically-unbiased state, in contrast to the mixed cellular milieu afforded by Bottenstein, in which non-oligodendrocytic and non-glial progenitor-derived phenotypes remain abundant.

11. As a result of these considerations, the selective propagation of mitotically-active oligodendrocyte progenitor cells from the neonatal rat brain, as taught by Bottenstein, does not predict the successful isolation of mitotic oligodendrocyte progenitor cells from postnatal or adult human brain tissue.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5/31/02

Steven A. Goldman, M.D., Ph.D.

PATENT
Docket No.: 19603/4230 (CRF D-2238A)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Richard Hutson
Serial No.	:	09/282,239	
Cnfrm. No.	:	To Be Assigned	Art Unit: 1652
Filed	:	March 31, 1999	
For	:	A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS	

THIRD DECLARATION OF STEVEN A. GOLDMAN UNDER 37 C.F.R. §1.132

Mail Stop
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor and Chief of the Division of Cell and Gene Therapy of the Department of Neurology, University of Rochester Medical Center, Rochester, New York, where I am the Glenn-Zutes Chair in Biology of the Aging Brain.

3. I am a named inventor of the above patent application.

4. I am familiar with U.S. Patent No. 5,726,145 to Bottenstein ("Bottenstein") and U.S. Patent No. 6,361,996 to Rao, et. al., ("Rao").

5. Rao et al. (Rao et al., "Glial-Restricted Precursors are Derived from Multipotential Neuroepithelial Stem Cells," *Dev. Biol.* 188:48-63 (1997), attached hereto as

Exhibit 1) clearly demonstrate the strong astrocytic bias of their cells, which generated few, if any, oligodendrocytes.

6. There are fundamental differences between the lineage restriction and potential of neonatal and adult oligodendrocyte progenitor cells (Noble et al., "The O2A (Adult) Progenitor Cell: A Glial Stem Cell of the Adult Central Nervous System," *Seminars in Cell Biol.* 3:413-22 (1992), attached hereto as Exhibit 2; and Windrem et al., "Fetal and Adult Human Oligodendrocyte Progenitor Cells Effectively Myelinate Dysmyelinated Brain," *Nature Medicine* (January, 2004) (in press), attached hereto as Exhibit 3, which has been accepted for publication (see attached Exhibit 4)). These biological differences between perinatal and adult progenitor cells were not recognized by Rao or Bottenstein, whose cells were restricted to neonatal rodent derivation.

7. Rat oligodendrocyte progenitors are neither biologically nor phenotypically homologous to human oligodendrocyte progenitor cells. Specifically, rat oligodendrocyte progenitors and oligodendrocytes both express the antigenic marker recognized by monoclonal antibody O4. In contrast, this marker is expressed by human oligodendrocytes and their immature forms, but NOT by mitotic oligodendrocyte progenitor cells (See Armstrong et al., "Pre-Oligodendrocytes from Adult Human CNS," J. Neurosci. 12: 1538-47, 1992; Gogate et al., "Plasticity in the Adult Human Oligodendrocyte Lineage," *J. Neurosci.* 14:4571-87 (1994), attached hereto as Exhibit 5; Kirschenbaum et al., "*In vitro* Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain," *Cerebral Cortex* 6: 576-89 (1994); Roy et al., "Identification, Isolation, and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells From the Adult Human Subcortical White Matter," *J. Neurosci.* 19: 9986-95 (1999) ("Roy, 1999"), attached hereto as Exhibit 6). As a result, human oligodendrocyte progenitor cells cannot be acquired through the use of O4 as a selection marker, and O4-defined human oligodendroglial cells cannot act as mitotically-competent progenitor cells. This is in sharp distinction to the rat brain, in which the use of this marker can identify oligodendrocyte progenitors. Neither Rao nor Bottenstein recognized the non-applicability of this marker to the separation of human oligodendrocyte progenitor cells. In humans, mitotic cells biased strongly towards the oligodendrocyte lineage are instead recognized by the antigenic phenotype $O4^-/PSA\text{-}NCAM^-/A2B5^+$, which comprise a distinct subpopulation in which the CNP2 promoter is transcriptionally activated (Roy, 1999; Windrem et al., "Progenitor Cells

Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Regions of the Rat Brain," *J. Neurosci. Res.* 69:966-75 (2002), attached hereto as Exhibit 7; Nunes et al., "Identification and Isolation of Multipotential Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain," *Nature Med.* 9: 439-47 (2003); attached hereto as Exhibit 8).

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12-17-03

Steven A. Goldman, M.D., Ph.D.

In vitro Neuronal Production and Differentiation by Precursor Cells Derived from the Adult Human Forebrain

Barry Kirschenbaum,[1] Maiken Nedergaard,[1] Axel Preuss,[1] Kaveh Barami,[1] Richard A. R. Fraser,[2] and Steven A. Goldman[1]

Departments of [1] Neurology and Neuroscience and [2] Neurosurgery, Cornell University Medical College, New York, New York 10021

It has traditionally been held that the adult brain is incapable of significant self-repair, due in part to its inability to generate new neurons. Nevertheless, rodents and birds have been found to harbor neural precursor cells in adulthood. We asked whether the adult human brain might retain such precursors, by culturing samples of temporal lobe under conditions permissive for neuronal differentiation, while exposed to ^{3}H-thymidine. Adult human temporal lobe cultures, derived from cortex, subcortex, and periventricular subependymal zone (SZ), were incubated for 7–28 d, stained for neuronal and glial antigens, and autoradiographed. Neuron-like cells were found in explant outgrowths and monolayer dissociates of SZ and periventricular white matter, but not cortex; they expressed neuronal antigens including MAP-2, MAP-5, NF, and N-CAM, and were GFAP$^-$. Neurons responded to K^+ depolarization with rapid and reversible increases in intracellular Ca^{2+}, with much greater increments than those noted in glia. Although most neurons were not ^{3}H-thymidine labeled, a small number of MAP-2$^+$ and MAP-5$^+$/GFAP$^-$ cells did incorporate ^{3}H-thymidine, suggesting neuronal production from precursor mitosis. Rare ^{3}H-thymidine$^+$ neurons were also found in cultures of subventricular white matter; in these, GFAP$^+$ astrocytic mitogenesis was common, while O4$^+$ oligodendrocytes, although the predominant cell type, were largely postmitotic. Thus, the adult human forebrain harbors precursor cells that retain the potential for neuronal production and differentiation *in vitro*.

Among adult mammals, forebrain neurogenesis is highly restricted, both spatially and phylogenetically (Altman and Das, 1966; Korr, 1980; Sturrock, 1982), and has not previously been found in primates (Rakic, 1985; Eckenhoff and Rakic, 1988). In contrast, neurogenesis is widespread and robust in the adult songbird telencephalon, which continues to generate neurons from mitotic ependymal or subependymal (SZ) precursor cells (Goldman and Nottebohm, 1983). [In the adult songbird, it remains unclear whether the precursor cell resides in the ventricular ependyma or one cell below, in the subependyma. As a result, our use of the abbreviation SZ in this article, for all species discussed, encompasses both the nominally defined ependymal and subependymal layers (Boulder Committee, 1970)]. Like the songbirds, lower vertebrates including both teleost fish (Anderson and Waxman, 1985) and lizards (Lopez-Garcia et al., 1988) have been shown to exhibit persistent neurogenesis in adulthood. We previously established a preparation by which neurogenesis could be studied in long-term explant cultures of the adult songbird forebrain (Goldman, 1990). In these explants, the number of neurons generated *in vitro* varied as an inverse function of the serum level, indicating that serum might harbor or induce factors that are antimitogenic for SZ precursor cells (Goldman et al., 1992b). On this basis, we postulated that the lack of neuronal production by non-neurogenic adult brain might result not from an absence of appropriate precursors, but rather from their tonic inhibition by either serum-borne or hormonally stimulated, locally derived agents.

Recent reports have demonstrated the presence of such neuronal precursor cells in cultures derived from adult brain. Reynolds and Weiss (1992) reported epidermal growth factor (EGF)-stimulated neurogenesis in cultures of the adult mouse striatum, while Richards et al. (1992) also described neuronal production in cultures of the adult mouse forebrain, under the influence of basic fibroblast growth factor (bFGF). Ronnett et al. (1990) observed the proliferation of neural cells derived from megalencephalic human brain; however, the transformation state and functional capability of these cells are unclear. Although the source of the neuronal precursors was not established in these reports, the characteristic pattern of ventricular zone neurogenesis in mam-

Cerebral Cortex Nov/Dec 1994;6:576–589; 1047-3211/94/$4.00

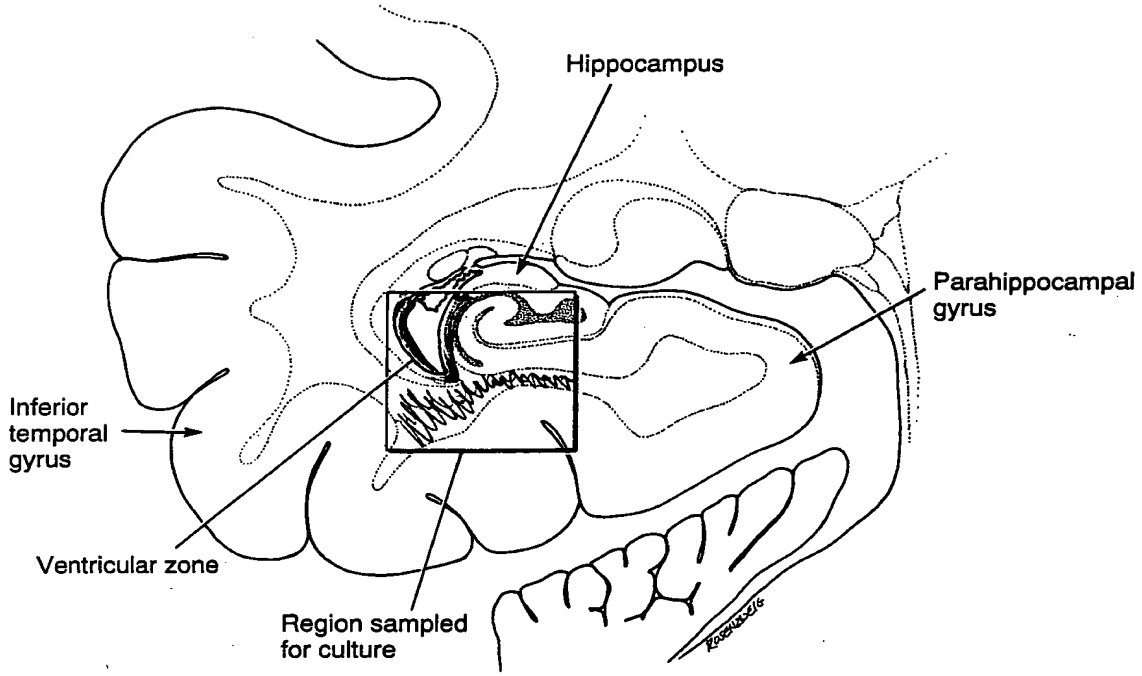


Figure 1. Adult human temporal lobe was obtained from refractory epileptics during anterior temporal lobectomy. This drawing shows a coronal section at a level roughly corresponding to the average posterior border of these resections. The borders of a typical inferior temporal lobe sample (typically from the nondominant hemisphere) are outlined as drawn. This includes the anterior aspect of the parahippocampal gyrus, temporo-occipital portion of the inferior temporal gyrus, and in some cases the hippocampus itself, all underlying the inferior aspect of the temporal horn of the lateral ventricle. From each resection, tissue pieces were dissected into cortical, subcortical, and SZ samples, the latter including both the ependymal surface and adjacent subependymal zone.

malian embryogeny, as well as in the adult avian brain, suggested that neuronal precursors may reside in the adult mammalian forebrain SZ. Indeed, SZ cells continue to divide in the adult mouse brain, but their progeny generally die within 24 hr after mitosis (Morshead and van der Kooy, 1992), although some degree of neuronal differentiation and survival may occur, particularly among cells destined for the olfactory bulb (Altman and Das, 1966; Kaplan and Hinds, 1977; Corotto et al., 1993; Luskin, 1993). In contrast, once removed into explant culture, the adult murine SZ demonstrates both the migration and differentiation of newly generated neurons (Lois and Alvarez-Buylla, 1993). These results suggested the persistence in adults of an SZ progenitor cell population, which remains actively neurogenic in selected groups and brain regions, but which more generally becomes vestigial, yielding short-lived or rare progeny. On this basis, we postulated that the adult human forebrain might harbor such vestigial precursor cells, which retain the capacity for neurogenesis when raised *in vitro*. To test this proposition, we sought evidence of neurogenesis in cultures of adult human temporal lobe. We report here that cells derived from the SZ and periventricular white matter of the adult human forebrain can indeed generate and differentiate into neurons in culture.

Aspects of this work have been reported previously in abstract form (Goldman et al., 1993; Kirschenbaum et al., 1993).

Materials and Methods

Tissue Samples

Adult human temporal lobe was obtained during anterior temporal lobectomy, done for the treatment of medically refractory epilepsy (n = 11 patients, 15–52 years old: four males, and seven females). No tissues were obtained from tumor of any origin, because of the potential danger in confusing proliferating neuroepithelial cells with neoplastic cells *in vitro*. No tissues were used that would otherwise have not been taken as a requirement of surgery. Tissue pieces were dissected into cortical, subcortical, and periventricular samples, the latter including the ependyma and subependymal zone (again, jointly denoted as SZ). The SZ was demarcated by ligature at resection, and dissected from subjacent white matter to a depth of approximately 300 μm (Fig. 1).

Culture Preparation

Each tissue sample was cut into roughly 0.3 mm^3 pieces, which were either cultured directly as explants on laminin, or dissociated for single-cell monolayer culture. Dissociate cultures were prepared by incubating pieces for 40 min in 0.25% trypsin, 1 mM EDTA at 37°C, with intermittent trituration. After pelleting and resuspension in media, cells were plated at roughly 2 × 10^5 cells/ml into either 35 mm petri dishes (0.7 ml/plate) or 24-well plates (0.4 ml/well), which had been coated with human fibronectin (GIBCO-Bethesda Re-

search Labs; 1 mg/cm²). Representative pieces were also cultured as explants, upon murine laminin (Sigma; 1 mg/cm²) according to described methods (Goldman et al., 1992b).

Media

We used a base medium that we had earlier found permissive for neurogenesis in adult avian explant cultures (Goldman et al., 1992), modified in that the base of Dulbecco's modified Eagle's medium/Ham's F-12 was prepared without either phenol red or glutamate, and the nonessential amino acid supplement also excluded glutamate and aspartate. In cultures of dissociated avian SZ, this medium supported neurogenesis with as little as 0.625% fetal bovine serum (FBS) (unpublished observation), although the present study reports only cultures raised at a serum concentration of 10%. Dissociated samples were pelleted and resuspended in this medium, and then plated as monolayer cultures. The medium was supplemented with 10% FBS, and dialyzed to MW 1000 Da. Selected cultures were also supplemented with EGF (Collaborative Research; 20 ng/ml). All cultures were given a complete change of media after 6 d *in vitro* (DIV), with half-volume changes twice weekly thereafter.

Immunocytochemistry

Cultures were incubated for 7–28 d and fixed, and each was probed with antibodies directed against one or two neuronal antigens, which included microtubule-associated protein-2 (MAP-2; Bernhardt and Matus, 1985), neurofilament (NF; Bignami et al., 1980), N-CAM (Edelman, 1984), and MAP-5 (Huber and Matus, 1984). Cultures were also probed with antibodies directed against a variety of astrocytic (glial fibrillary acidic protein, GFAP), oligodendrocytic (O1, O4), pro-oligodendrocytic (A2B5 and G_{D3}, as well as O4), and microglial (CD68) antigens, to differentiate among glia (Kelly et al., 1988; Vaysse and Goldman, 1990).

Neurons were defined as those cells with typical multipolar morphology and immunoreactivity for N-CAM, MAP-2, NF, or MAP-5, but not GFAP. We used the following antibodies for neuronal identification in this study: mouse anti-N-CAM IgG (1:25; Sigma, clone OB11); rabbit anti-neurofilament serum (1:100; Dr. D. Dahl); rabbit anti-MAP-2 (1:100; Dr. I. Fisher) (Fisher et al., 1987); mouse anti-MAP-5 (1:100; Sigma, clone AA6). The protocol used for detecting each of these antigens was as previously described for MAP-2 (Goldman, 1990), with secondary antibodies appropriate to the species and idiotype of each primary antibody.

Glial cell types were characterized on the basis of previously defined criteria (Cameron and Rakic, 1991). Oligodendrocytes were defined as immunoreactive for the O4 antigen (Bansal et al., 1989), whether $GFAP^+$ or $GFAP^-$. Astrocytes were identified by their expression of GFAP, except for those that coexpressed GFAP and O4, which were classified as oligodendrocytic. Microglia were characterized by the CD68 antigen (Kelly et al., 1988), and pre-GFAP and/or O4 glia as either A2B5 (Eisenbarth et al., 1979) or G_{D3} immunoreactive (Goldman et al., 1984). To identify these glial types, we used the following antibodies: mouse monoclonal antibodies O4 and O1 IgM (1:50; Dr. R. Bansal), mouse anti-G_{D3} IgG (1:25; clone R24, Dr. J. Goldman), mouse A2B5 IgM (1:14; Dr. K. Fields), anti-GFAP IgG (1:100; Sigma, clone GA5), and mouse anti-microglial CD68 IgG (1:100; Dako, clone EBM11). The protocols used for each of these probes were also as previously described (Kelly et al., 1988; Vaysse and Goldman, 1990; Bansal et al., 1989). All surface antigens (O4, O1, G_{D3}, A2B5, CD68) were probed on live cells, which were fixed after antibody exposure for 10 min with cold, 2% paraformaldehyde. Skeletal antigens were probed in 4% paraformaldehyde-fixed, saponin-permeabilized cultures. All antigens were then detected using fluorescent secondary antibodies at 1:50–100.

We verified the immunoreactivity and optimal titer of each antibody in a series of embryonic rat forebrain cultures (data not shown). A separate set of control cultures, prepared to assess nonspecific immunostaining, were exposed to either mouse IgG (10 mg/ml; Sigma), mouse anti-agrin IgG (1:100; Dr. E. Godfrey), or normal rabbit serum (1:100; GIBCO), followed by appropriate secondary antibodies. None of these controls displayed significant immunostaining.

^{3}H-Thymidine Labeling

The uptake of ^{3}H-thymidine by antigenically defined neurons was used as an index of antecedent precursor cell mitosis *in vitro*. ^{3}H-thymidine (0.2 μCi/plate, from 1 mCi/ml stock; 5 Ci/mM, Amersham) was added 6 hr after culture preparation, so that S-phase initiation of labeled cells would have occurred *in vitro*. Cultures were exposed to ^{3}H-thymidine during their first 6 DIV, after which a complete medium exchange removed residual isotope. As noted, all cultures were fixed 7–28 d after establishment, and then immunostained and autoradiographed. Autoradiography was performed as previously described (Goldman, 1990; Goldman et al., 1992b), after which the percentage of ^{3}H-thymidine$^+$ neurons in each culture was calculated, and used as an index of *in vitro* neurogenesis. ^{3}H-thymidine-labeled cells were defined as having ≥10 silver grains over their nuclei (background averaged <1 grain/10^2 μm²). Labeled cells were presumed to have been in S-phase at the time of ^{3}H-thymidine exposure, and to have arisen by the *in vitro* mitosis of parental progenitors.

Calcium Imaging

Cells were challenged with a depolarizing stimulus of 60 mM K^+, during which their cytosolic calcium levels were observed. To this end, cultures were loaded with 10 μM fluo-3 acetomethoxyester (fluo-3 AM; Molecular Probes) for 1 hr at 37°C. A Bio-Rad MRC600 confocal scanning microscope, coupled to Olympus IMT-2 inverted microscope, was used to image the fluo-3 signal. Excitation was provided by the 488 nm line of a 25 mW argon laser, filtered to ≤0.1% by neutral density filters. Emission was long-pass filtered (515 nm) and detected with the confocal set to its maximal aperture (7 mm). Images were acquired every 1–5 sec and recorded on a Panasonic TQ-2028F optical disk

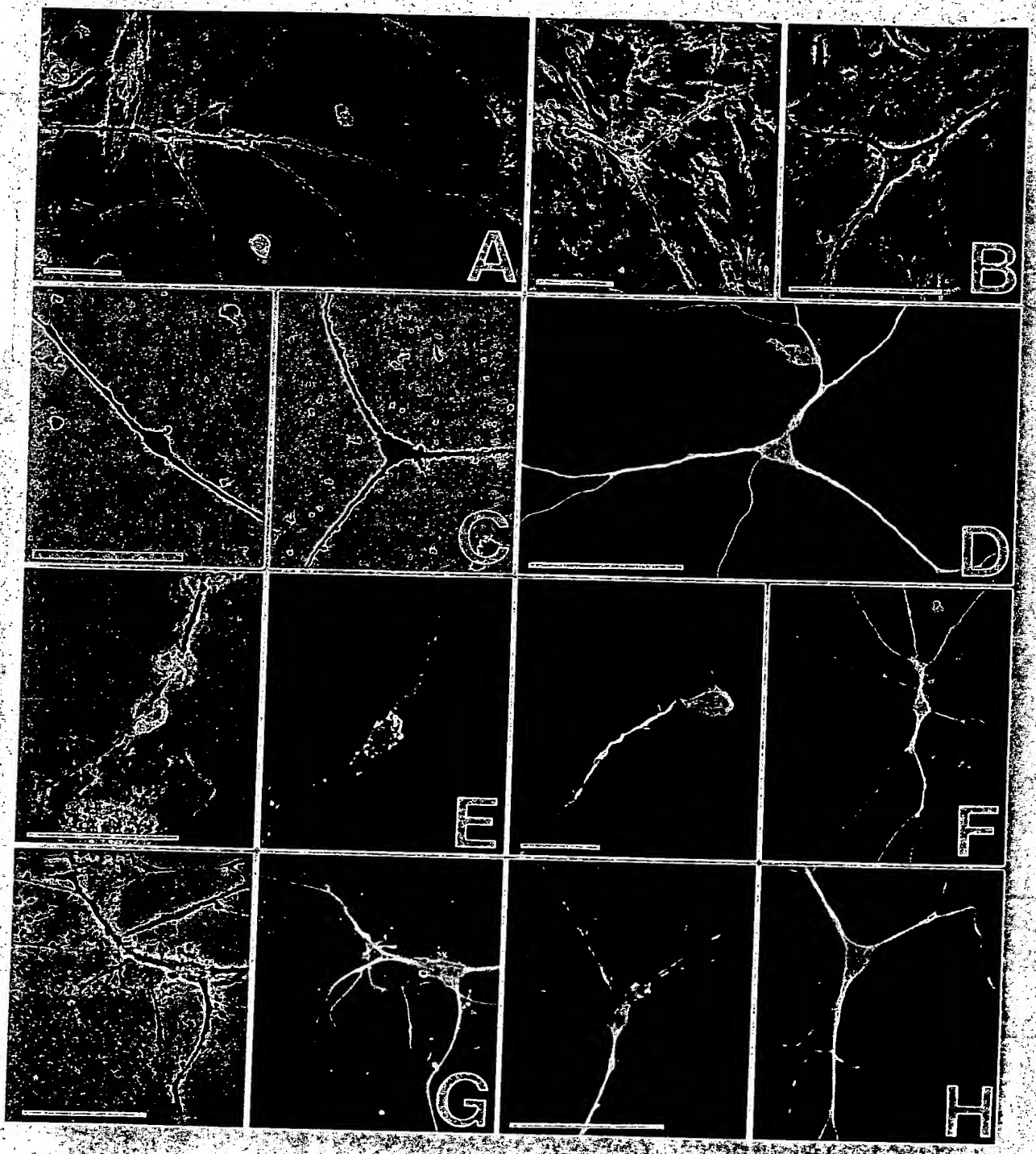
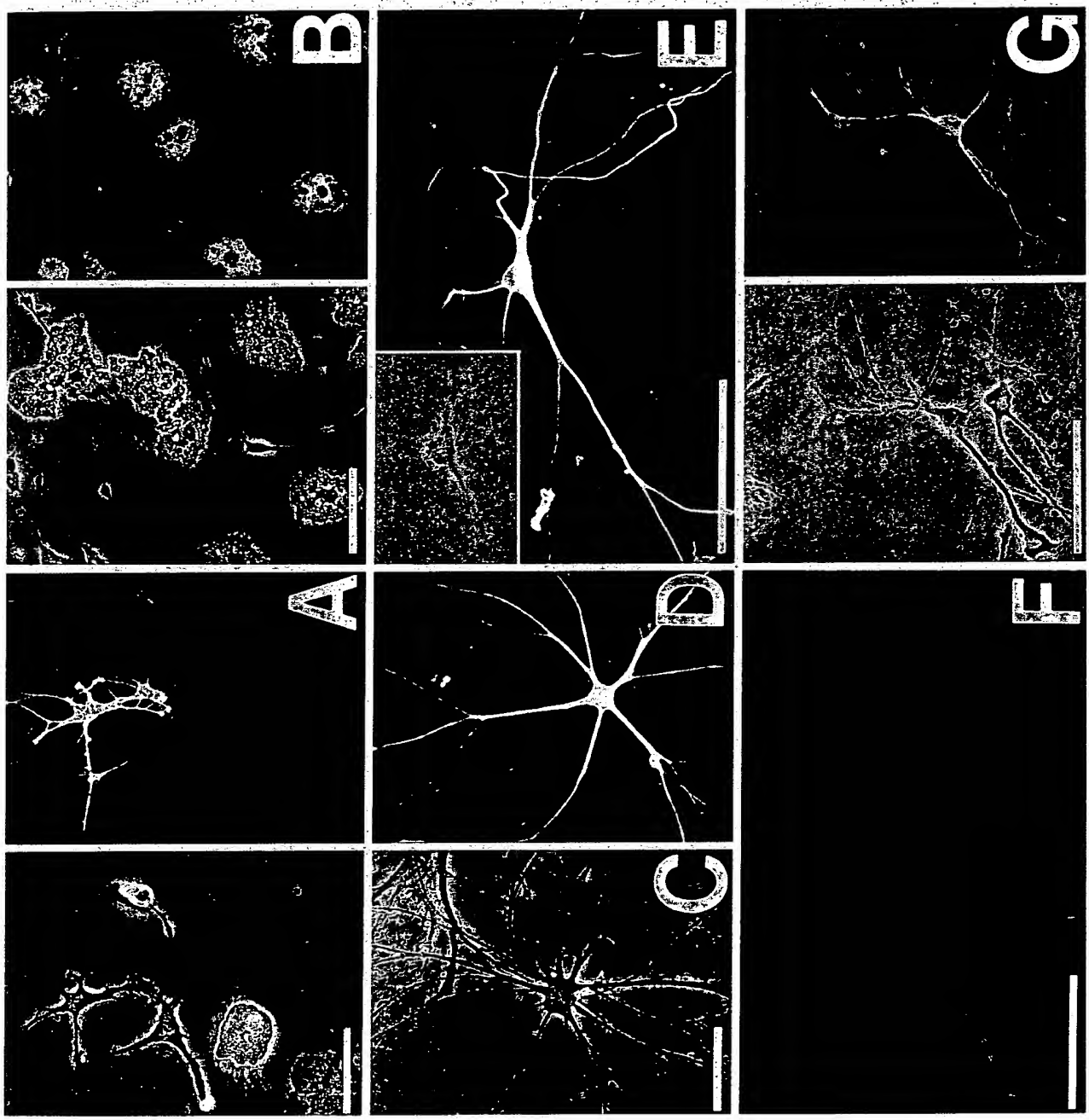


Figure 2: Neuronal differentiation in cultures of adult human temporal lobe. Neurons were found in small numbers in both explant outgrowths and dissociates of adult human SZ, and rarely, subcortical white matter. *A* and *B* display explant outgrowths derived from temporal horn SZ, in which presumptive neurons are seen upon a layer of flat astrocytes after 19 d *in vitro* (DIV). *C* shows two relatively immature cells obtained from dissociated temporal SZ, fixed and stained for MAP-2 after 8 DIV. *D* shows another MAP-2$^+$ neuron, found in a subcortical dissociate after 18 DIV. This sample may have harbored residual SZ, but our data allow the possibility that rare precursors with neuronal potential persist in the subcortical white matter as well as the SZ. *E* shows an N-CAM$^+$ cell found in another culture of dissociated temporal subcortex, at 18 DIV. Small N-CAM$^+$ cells like these were common in subcortical dissociates. The vast majority developed into O4$^+$/N-CAM$^-$ pro-oligodendrocytes, but a small number retained N-CAM expression, failed to develop oligodendrocytic antigenicity, and instead developed neuronal morphology and antigenicity. *F* shows two such N-CAM$^+$ cells, found in a subcortical dissociate after 18 DIV. *G* shows phase and fluorescent images of an NF$^+$ neuron, found in an SZ dissociate after 18 DIV, while *H* displays two images of MAP-5$^+$ cells after 15 DIV. Scale bars, 50 μm.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recorder. Relative changes in fluorescence were calculated and normalized against baseline fluorescence by $\Delta F/F$ (Connor et al., 1987), and background counts were subtracted from all experiments. Each experiment was carried out at 25°C in HBSS, with 60 mM K^+ exchanged for 60 mM Na^+ in the potassium-depolarizing solution.

Results

Antigenic Identification

Among the 11 brain samples, eight included ventriculotomy and hence SZ tissue. One set of cultures failed technically, and one set was lost during processing, giving us six sets of SZ cultures. Fiber-projecting, neuron-like cells were found in both SZ explant outgrowths and dissociates. These cells were typically phase bright at low power, and phase dark at higher magnification. Unlike cocultured astrocytes, these cells had no large cytoplasmic inclusions, their cell bodies lacked lamellopodial extensions onto the substrate, and they had only two or three primary processes, which ramified distally (Fig. 2). Representative examples of these neuron-like cells were found to express MAP-2, MAP-5, N-CAM, or NF, while failing to stain concurrently for GFAP. Indeed, MAP-2, MAP-5, N-CAM, and/or NF^+ neuron-like cells were each found in cultures taken from at least three of the six SZ-containing samples (Fig. 2). (Not all antigens were sought in plates derived from each SZ sample.) The antigenicity of these cells suggested that they were in fact phenotypic neurons.

Subependymal Zone Derivatives

We examined the postulate that these neurons arose from SZ progenitors, by assessing neuronal outgrowth from explants taken from the temporal SZ (a region that in the adult human is sparse in neurons), relative to that observed in temporal subcortical and cortical explants. Among six brains from which temporal SZ explants were prepared, four produced cellular outgrowth and three displayed morphologically neuron-like cells migrating upon subjacent astrocytes and/or flat glioblasts (Fig. 2). Representative explants from these brains were stained for MAP-2, MAP-5, or N-CAM; explants from one were also subjected to physiological analysis (see below). While neurons were found in the outgrowth from SZ explants, as well as in SZ dissociates (Fig. 2), no similar outgrowth was observed from a total of over 300 explants taken from the temporal neocortex (seven tissue samples, each yielding 24–36 explants dispersed among four to six culture plates) and subcortex (four samples, again yielding 24–36 explants each). Unfortunately, meaningful quantification of neuronal outgrowth from SZ explants was hindered by the variability in the amount and location of SZ obtained from each brain, which was dictated by the surgical procedure. A likely source of additional variability may have been the heterogeneous distribution of potential precursor cells within the SZ (Luskin, 1993).

Subcortical Phenotypes

In subcortical dissociates, in contrast to subcortical explants, rare neurons were noted in plates derived from two of the tissue samples, although no subcortical culture had more than 10 (as in the SZ samples, neurons were characterized antigenically as either $MAP\text{-}2^+/GFAP^-$, $MAP\text{-}5^+/GFAP^-$, or $N\text{-}CAM^+$; see Fig. 2). Not surprisingly, more fiber-bearing cells were harvested from subcortical white matter than from SZ, as a result of the white matter's enrichment in $O4^+$ oligodendrocytes and fibrous astrocytes. Since our subcortical cultures generally contained at least 10^3 fiber-bearing cells/plate, when neurons were found, they constituted <1% of the fiber-projecting cell population. Far more common were fibrous astrocytes and oligodendrocytes, which were variably $GFAP^+/O4^-$ and $GFAP^\pm/O4^+$, respectively. Astrocytes were ubiquitous and pleomorphic (Fig. 3*C–F*), but the predominant (>80%) fiber-bearing cell of the subcortical dissociates was the $O4^+$ oligodendrocyte (Fig. 3*G*); the latter was only rarely noted in the SZ dissociates and explants. Both $GFAP^+$ and $GFAP^-$ examples of $O4^+$ cells were noted.

Most of the cells found in these dissociate cultures were not fiber bearing at all, and were readily distinguished from neurons. These cells typically comprised the majority of each culture even after 6 DIV, although their relative proportions varied as a function of the culture conditions and region sampled (data not shown). These cells included capillary endothelia, flat $GFAP^-$ cells of uncertain phenotype, small and undeveloped $GFAP^+$ astrocytes, $EBM11/CD68^+$ amoeboid

←

Figure 3. Glial phenotypes. Glia were pleomorphic in these cultures, but remained identifiable on the basis of their GFAP and/or O4 expression, lack of MAP-2 and NF immunoreactivity, minimal and/or transient MAP-5 and N-CAM expression (by oligodendrocytes only), and limited calcium response to high K^+. Both SZ and cortical dissociates were composed primarily of amoeboid microglia, endothelia, flat astroblasts, and $GFAP^+$ astrocytes. In *A*, the presence of immature glia or their precursors was suggested by the presence of G_{D3}^+ cells; a single G_{D3}^+ is shown here, among other G_{D3}^- glia and microglia, after 12 DIV. *B* shows corresponding phase and fluorescent views of microglia found with rare fiber-bearing cells in an SZ culture after 7 DIV. This culture was immunostained for microglial CD68 with monoclonal antibody EBM11. *C* shows an example of a mature astrocyte in an SZ culture after 27 DIV. Cultures of dissociated subcortex yielded $GFAP^+$ cells of a different morphology, with branching arbors of thin fibers, likely fibrous astrocytes. *D* and *E* show two subcortical astrocytes stained for GFAP, at 18 DIV; these cells were of strikingly "neuron-like" morphology. Some (e.g., *E, inset*) incorporated ^{3}H-thymidine, and were presumably generated *in vitro*. Subcortical cultures also harbored fiber-bearing cells of oligodendrocytic lineage. *F* shows the many fiber-bearing cells of a subcortical dissociate at 22 DIV. The majority were $GFAP^-$, as in *F*, in which only three $GFAP^+$ cells are seen among a field of smaller, process-bearing cells. Although these $GFAP^-$ subcortical cells included rare $MAP\text{-}2^+/MAP\text{-}5^+$ neurons (see Figs. 2, 4), most expressed the oligodendrocytic lineage marker O4. *G* shows $O4^+$ oligodendroglia (Texas *red* fluorescence) found in a subcortical dissociate after 14 DIV, with admixed $GFAP^+$ astrocytes (*green* fluorescence). The antigenic phenotype of the $O4^+$ cells ($O4^+/O1^\pm/A2B5^-/G_{D3}^-/MAP\text{-}2^-/MAP\text{-}5^{weak+}$) was consistent with that of pro-oligodendrocytes (Armstrong et al., 1992). These may have arisen from dedifferentiated oligodendrocytes, or from postmitotic oligodendroglial precursors. Scale bars, 50 μm.

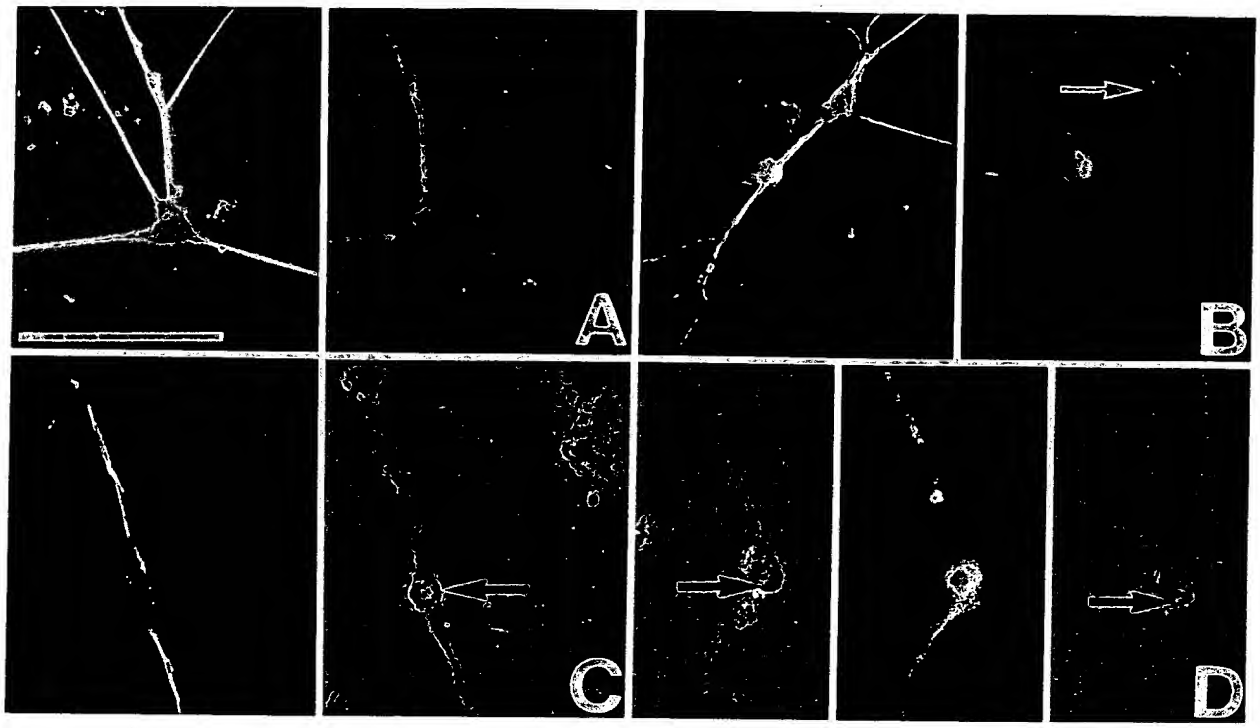

Figure 4. Neurogenesis *in vitro*. *A–C* show MAP-5$^+$ cells observed in dissociate cultures, after 15 DIV. *A* and *B* were derived from subventricular white matter, and *C* from nominal SZ; all plates were fixed after 15 DIV. The indicated cells (*arrows*) have each incorporated ^{3}H-thymidine *in vitro*, suggesting their origin from precursor mitosis. *D* shows a pair of adherent MAP-2$^+$ cells found in an SZ dissociate at 14 DIV, only one of which (*arrow*) has incorporated ^{3}H-thymidine. Scale bar, 50 μm.

and ramified microglia (Fig. 3*B*), and rare (≤10 cells/plate) A2B5$^+$ and G_{D3}^+ cells (Fig. 3*A*).

Neurogenesis

Among antigenically defined neurons (MAP-2$^+$, MAP-5$^+$, N-CAM$^+$, or NF$^+$), autoradiography revealed ^{3}H-thymidine$^+$ cells in samples derived from three brains, indicating the origin of these cells from precursor mitosis *in vitro* (Fig. 4). Examples of ^{3}H-thymidine$^+$ cells expressing each of these neuronal antigens were found. However, the overall yield of ^{3}H-thymidine$^+$ neurons was low: in one representative sample of dissociated SZ, of 2×10^5 cells plated into each of three petri dishes, a total of <500 fiber-bearing cells (<0.25% of the initial cell sample) were found in the three plates after 14 DIV. The vast majority of the initial cell sample had died, and as noted above, the survivors included a mixture of flat uncharacterized cells, small stellate astrocytes, capillary endothelial cells, and microglia. Among the SZ-derived, fiber-bearing cells of neuron-like morphology, 393 were actually GFAP$^+$, while 56 were MAP-2$^+$. Of the 393 GFAP$^+$ cells, 79 (20%) were ^{3}H-thymidine$^+$, while of the 56 antigenically verified neurons, only six (11%) had incorporated ^{3}H-thymidine$^+$ *in vitro*.

Gliogenesis

^{3}H-thymidine-incorporating GFAP$^+$ astrocytes were frequently noted in cultures of all three sampled regions, including SZ, subcortex, and cortex (e.g., Fig. 3*E*). Similarly, ^{3}H-thymidine$^+$ microglia were frequently noted (data not shown). In contrast, two mitotically distinct classes of O4$^+$ cells were noted. The first was characterized by relatively ovoid, 8–10 μm cell bodies that projected a variable number (two to nine) of relatively thin, short fibers, which frequently branched within <100 μm of the soma. These cells were intensely O4$^+$, and variably GFAP$^+$ (Fig. 3*G*). These O4$^+$/GFAP$^\pm$ cells were mitotically quiescent; among a sample of 8044 such cells, culled from four plates of subcortical white matter (2011 ± 858.6 O4$^+$ cells/plate, mean ± SD), *none* incorporated ^{3}H-thymidine *in vitro*, despite the frequent observation of ^{3}H-thymidine-labeled astrocytes in the same plates. These cells likely correspond to the pro-oligodendrocytes previously characterized by Armstrong et al. (1992). In contrast, a second, comparatively uncommon category of O4$^+$ cells was characterized by a larger (15–25 μm), flatter, and more substrate-apposed soma; each cell projected several relatively thick, long and tapering, unbranched processes. These cells constituted <1% of the O4$^+$ population, and frequently incorporated ^{3}H-thymidine. The ontogeny and fate of these O4$^+$/^{3}H-thymidine$^+$ cells are now being evaluated separately (Kirschenbaum and Goldman, unpublished observation).

Cell-Specific Calcium Responses

To assess the functional capability of observed neurons, 24 cultures were loaded with the calcium-sensi-

tive dye fluo-3, and exposed to 60 mM K^+ during confocal microscopy, to detect depolarization-induced Ca^{2+} increments (Fig. 5). Of these cultures, five were derived from SZ explants, and the remainder from either subcortical ($n = 15$) or cortical cultures ($n = 4$). Glial responses to 60 mM K^+ were minimal; of a pooled sample of subcortical and cortical glia (selected for fiber-bearing astrocytes and pro-oligodendrocytes), an average increment of 25 ± 3.5% (mean ± SE, $n = 83$) was noted in cytosolic calcium following K^+ stimulation. In contrast, neurons displayed a rapid and reversible four-fold elevation in cytosolic calcium signal in response to K^+ (402 ± 107.0%, $n = 5$; $p < 0.0001$); consistent with the expected activity of neuronal voltage-gated calcium channels (Connor et al., 1987; Hockberger et al., 1987).

Discussion

The present results suggest that the adult human forebrain harbors precursor cells that retain the potential for neuronal production and differentiation *in vitro*. These cells appear to reside predominantly in the SZ, and in this study were found in samples derived from the ventrolateral aspect of the anterior temporal horn of the lateral ventricle. The adjacent subventricular white matter was found to harbor glial precursors, in that ^{3}H-thymidine$^+$ examples of both $GFAP^+/O4^-$ and $GFAP^+/O4^+$ cells were identified, as well as a distinct population of postmitotic, $O4^+/GFAP^-$ fibrous cells similar to those described as pro-oligodendrocytes (Armstrong et al., 1992).

Limitations of Antigenic Analysis

Any antigenic determination of phenotype among brain cells is limited by the lack of absolute cell-type specificity of currently identified neuroectodermal antigens. In particular, several antigenic markers previously considered prototypic of neuronal phenotype are actually expressed in developmentally restricted time windows by glia as well as neurons. Thus, whereas MAP-2 and MAP-5 are typically characterized as neuronal proteins (Huber and Matus, 1984; Bernhardt and Matus, 1985), reactive white matter astrocytes have been shown to express MAP-2 transiently (Geisert et al., 1990), while MAP-5 can be expressed by $O4^+$ pro-oligodendrocytes in culture (Vouyiouklis and Brophy, 1993). [In this regard, we found that MAP-5 expression by $O4^+$ pro-oligodendrocytes, although generally present, was far less than, and readily distinguished from, that demonstrated by $MAP\text{-}5^+/O4^-$ cells in human SZ and subcortical cultures; similarly, oligodendrocytic MAP-5 immunoreactivity was far less than that displayed by cocultured neurons in rat forebrain control cultures (data not shown).] Like MAP-5, N-CAM is expressed by early oligodendrocytes as well as by neurons (Bhat and Silberberg, 1986), and neurofilament may also be transiently expressed by astrocytes in culture (Galileo and Linser, 1992). Thus, no one of these markers is alone sufficient to define neuronal phenotype. As a result, we used a panel of neuron-selective antigens for neuronal identification in these cultures. Although individual cultures were typically probed with only one or two antibodies, matched cultures were probed for alternative neuronal antigens; most plates were probed concurrently with anti-GFAP, or in some cases O4. We required concurrent demonstration of positive neuronal antigenicity and negative glial staining for defining neuronal identity. In addition, in selected cultures of both explanted SZ and dissociated subventricular white matter, we obtained antigen-independent verification of neuronal presence and function, by assessing the cytosolic calcium responses to depolarizing stimuli of individual fiber-bearing cells.

Physiological Characterization

Although K^+-induced calcium responses have been described among cortical astrocytes in culture (MacVicar et al., 1991; Fatatis and Russell, 1992), depolarization-induced calcium increments in glia have been of lesser magnitude than those noted in neurons under analogous conditions (Connor et al., 1987; Hockberger et al., 1987). Indeed, only under conditions of cAMP stimulation, using cultures of confluent astrocytes raised over a month *in vitro*, have astrocytes been reported to display significant K^+-evoked Ca^{2+} increments (MacVicar et al., 1991). In that same study, astrocytes raised under conditions more analogous to those of our present study; that is, subconfluent cells maintained for shorter periods of time *in vitro*, and not exposed to exogenous cAMP, displayed only minor elevations, and often decrements, in K^+-evoked cytosolic Ca^{2+}. Nevertheless, no previous study of which we are aware has directly compared the depolarization-induced Ca^{2+} responses of astrocytes, oligodendrocytes, and neurons in single cultures.

In order to control for the possibility of some cultured astrocytes displaying neuron-like Ca^{2+} responses, we exposed mixed neuronal and glial cultures of embryonic (E16) rat forebrain, after 18 DIV, to 60 mM K^+ and correlated their Ca^{2+} responses with post hoc immunocytochemistry for either MAP-2 or GFAP (Nedergaard and Goldman, unpublished observation). These cells were raised under the same conditions as their adult human brain counterparts. We found that a sample of 86 antigenically verified $MAP\text{-}2^+$ neurons, randomly sampled from five cultures, exhibited a mean Ca^{2+} increment of 267 ± 106% (mean ± SD). In contrast, a cocultured population of 126 $GFAP^+$ astrocytes displayed a mean Ca^{2+} elevation of 0.8 ± 17% (the low mean increment reflected the many astrocytes whose Ca^{2+} levels fell, by as much as 50%, in response to K^+). The response ranges of these two rat brain cell types were nonoverlapping, and the difference between them significant to $p < 0.001$. These data suggest the validity of the physiological criteria used for cell-type identification in our adult human brain cultures. Nonetheless, the response characteristics of adult cells may differ from their embryonic counterparts. Thus, even though the K^+-induced Ca^{2+} responses of cells such as that displayed in Figure 5 are consistent with neuronal phenotype, single-cell recording will be needed to establish whether these cells meet a more stringent criterion of neuronal func-

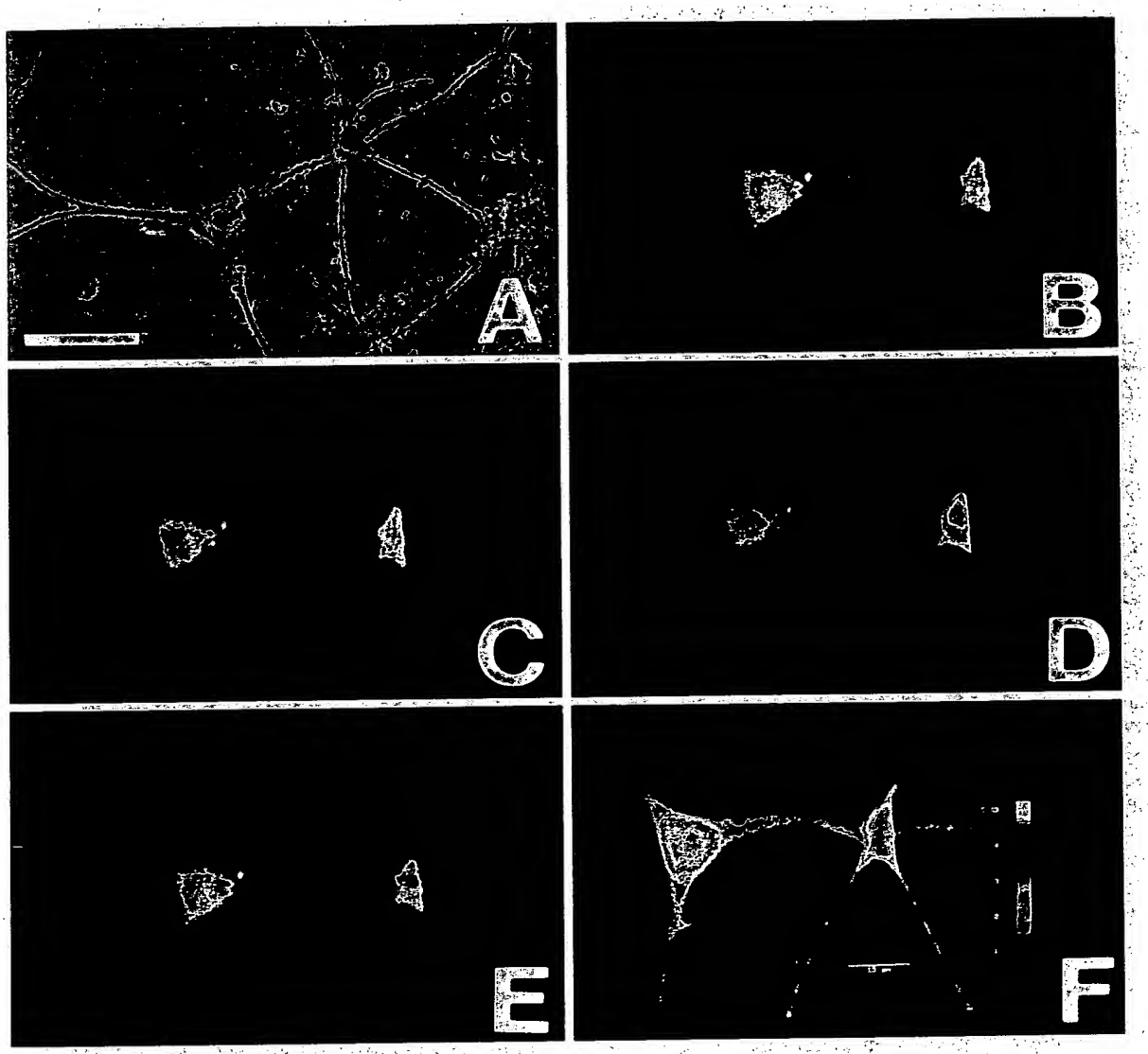

Figure 5. Calcium increments to depolarization by neurons in temporal SZ explants. Selected cultures were challenged with high K^+, to seek evidence of neuron-like depolarization-induced increments in cytosolic calcium (Ca^{2+}_i). In this plate, a temporal SZ culture was tested after 28 DIV, after loading with the Ca^{2+}-sensitive dye fluo-3. *A* shows a phase micrograph of two adjacent cells, one neuron-like and the other astrocytic. *B* shows their baseline levels of Ca^{2+}_i, as viewed by confocal microscopy with laser scanning at 488 nm. *C* shows the same two cells within seconds after exposure to 60 mM K^+. The neuron-like cell increased its Ca^{2+}_i rapidly and reversibly, in contrast to the cocultured astrocytes. In *D*, with the addition of tetrodotoxin (TTX; 1 μM; Sigma), K^+ stimulation yielded a greater than sixfold rise in neuronal cytosolic Ca^{2+}_i, while astrocytic Ca^{2+}_i increased less than twofold. The depolarization-induced Ca^{2+} increment of this cell suggested its neuronal phenotype, as did the TTX accentuation of its Ca^{2+}_i response; the increased density of TTX-sensitive Na^+ channels in neurons, relative to glia, would have been expected to yield a neuron-selective enhancement of the K^+-stimulated Ca^{2+}_i response by TTX (Ritchie and Rogart, 1977; Howe and Ritchie, 1990). (The TTX accentuation of the K^+-stimulated Ca^{2+} increment may also result from TTX's inhibition of depolarization-induced Na^+ influx; in the presence of TTX, when neuronal voltage-activated calcium channels open in response to K^+ stimulation, the Ca^{2+} cation can follow the ambient electrochemical gradient, unopposed by concurrent Na^+ entry.) In *E*, upon withdrawal of K^+ from the medium, each cell returned to its resting Ca^{2+}_i level. *F* was taken after addition of the calcium ionophore lasalocid (50 mM; Sigma), added as a positive control in order to maximize Ca^{2+} entry in both cells. These results suggested the activity of voltage-gated calcium channels in the adult-derived neurons. Scale bar, 25 μm.

tion, that of firing repetitive action potentials (Goldman and Nedergaard, 1992).

Source of the Neuronal Precursor Cells

At least some of the neuronal precursor cells reside within the SZ of the temporal horn of the lateral ventricle. These cells may represent vestiges of the embryonic SZ neuroepithelium, which retain the capacity for neuronal differentiation, and to some extent mitotic neurogenesis, when removed into tissue culture.

More difficult to explain were the rare neurons found in subcortical dissociates. These were so infrequent as to be of unclear significance; most likely, they derived from SZ inadvertently admitted into the subcortical sample during dissection. Alternatively, these neurons arose from ependymal/subependymal rests lying ectopically in the subventricular white matter

(Larroche, 1977). Similarly, since the surgical samples taken often included the parahippocampal and hippocampal gyri, these may have been included inadvertently in the subcortical dissections. In particular, any admixed hippocampal tissue would include the fascia dentata, whose granule neurons have been shown to undergo persistent turnover in adult rodents (Altman and Das, 1966; Kaplan and Hinds, 1977). Thus, some or all of the ^{3}H-thymidine$^+$ neurons found in the subcortical dissociates may have derived from mitotic dentate neuroblasts, rather than from contaminating SZ.

The observed neurons, both of our SZ and subcortical samples, might also have derived from clusters of granule neurons that lie ectopically in the basal forebrain subcortex. These cells, designated islands of Calleja, have been described across mammalian genera, including humans, and have been typically associated with subcortical olfactory pathways (Meyer et al., 1989). Interestingly, two studies have recently reported a population of newly generated neurons in the postnatal rodent basal forebrain, which migrates along a spatially restricted pathway to the olfactory bulb (Corotto et al., 1993; Luskin, 1993). It is possible that the seemingly ectopic aggregations of granule neurons in the adult human forebrain are homologous to this novel population of rodent forebrain neurons, and that our subcortical dissections may have included neuronal precursors lying within this pathway. Finally, the subcortically derived neurons might be the progeny of oligodendrocytic precursors resident within the white matter, which might retain the potential to generate neurons; such a bipotential neuronal-oligodendrocyte precursor has already been described in the embryonic rodent brain (Williams et al., 1991; Vescovi et al., 1993), although no counterpart has yet been identified in adulthood.

Whatever their geographic and cellular sources, most of the neurons identified in these cultures were unlabeled by ^{3}H-thymidine. These cells may have derived from the neuronal differentiation of precursors rendered postmitotic by the culture conditions. Alternitively, if the neurons identified in these cultures had simply represented resident neurons that had survived dissociation or explantation and regenerated neurites *in vitro,* then one might suppose that the neocortical cultures, which harbored by far the greatest number, density, and variety of neuronal phenotypes, would have yielded the most neurons. To the contrary, however, antigenically defined neurons were absent in our cortical explant outgrowths, which typically yielded only astrocytes and microglia, and rare O4$^+$ oligodendrocytes. Instead, the neurons identified in these cultures were limited to SZ and some periventricular white matter samples, precisely those regions in which neurons are scarce *in vivo.*

In our similar prior study of adult avian brain cultures raised in high-serum (Goldman, 1990), only those explants derived from neurogenic regions of the SZ displayed neuronal outgrowth, while striatal parenchymal explants failed to do so. Furthermore, prelabeling the mitotic precursor population with ^{3}H-thymidine *in vivo* revealed that most, if not all, of the neurons in these outgrowths were newly generated. Similar findings were obtained in explant cultures of the adult mouse (Lois and Alvarez-Buylla, 1993) and rat brains (Goldman and Kirschenbaum, 1994). In these rodent preparations, neuronal outgrowth was limited to explants derived from the SZ, and the emigrating neurons could be prelabeled with ^{3}H-thymidine given *in vivo,* prior to sacrifice. Thus, in both the adult songbird and rodent preparations, explant cultures selected precisely for those newly generated, migratory neurons that had just arisen from mitotic precursors.

Our present study was limited by our inability to prelabel the mitotic cells of the adult human brain prior to tissue resection and culture. Nonetheless, on the basis of (1) these parallel studies in other species, (2) the restriction of adult human neuronal outgrowth in the present study to SZ and peri-SZ subcortical explants, (3) the complete absence of neuronal outgrowth from the human cortical explants that we prepared, and (4) the occasional neurons found in human SZ and subcortical cultures exposed to ^{3}H-thymidine *in vitro,* it is likely that the ^{3}H-thymidine$^-$ neurons of these cultures were not just resident neurons that survived *ex vivo.* Rather, they appear to have derived from precursors that embarked upon neuronal differentiation *in vitro,* once removed from the tissue environment.

These precursors may have been mitotically competent *in vivo,* but lacked mitotic stimulation *in vitro,* or indeed may have been actively removed from the cell cycle by exposure to differentiation agents in culture. Alternatively, they may have derived from a resident pool of undifferentiated but postmitotic precursor cells. Evidence for the latter has been described in cultures of the postmitotic chick tectum, in which mitotically quiescent precursor cells with neuronal potential persist, and can be induced toward terminal neuronal differentiation by local neuronal depletion (Galileo et al., 1991).

The Culture Environment

In this study, we sought to increase the likelihood of finding *any* residual neural precursors in the adult brain, by raising the cultures in a high-serum medium that would optimally support neuronal differentiation, rather than precursor proliferation. Although prior reports have demonstrated that both EGF and bFGF may stimulate the division and proliferation of neural precursors in culture (Gensburger et al., 1987; Reynolds and Weiss, 1992; Richards et al., 1992; Kilpatrick and Bartlett, 1993; Ray et al., 1993), neuronal mitogenesis in these preparations appeared to require either media containing little or no serum (Reynolds and Weiss, 1992), or media supplemented with mitogen (bFGF; Richards et al., 1992). In contrast, neuronal *differentiation* from adult-derived precursor cells has been most robust in cultures raised at relatively high serum levels, in the absence of exogenous mitogenic agents, in both rodent (Lois and Alvarez-Buylla, 1993) and avian (Goldman, 1990) preparations. Indeed, when the effect of serum concentration upon adult neuronal mi-

togenesis was tested directly in explant cultures of adult finch forebrain, the neuronal ^{3}H-thymidine labeling index was found to be inversely proportional to the ambient serum level (Goldman et al., 1992b). Not surprisingly then, in the presence of the relatively high serum concentration used in this study (10%), no specific induction of neuronal mitogenesis by EGF was noted. Yet, the relatively selective detection of neurons in the SZ dissociates and explant outgrowths, and the incorporation by some of these cells of ^{3}H-thymidine, strongly suggested the existence of precursor cells with neuronal potential in these cultures. To the extent that these cells are premitotic, one might predict that they should be passageable for serial propagation. Although conditions permissive for the serial passage of embryonic rodent neural precursor cells have recently been described (Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1993; Ray et al., 1993), conditions appropriate for the sequential clonal expansion and neuronal differentiation of adult human precursor cells remain to be defined.

Lineage Potential of Single Precursor Cells

The presence of ^{3}H-thymidine$^+$ neurons and glia in these cultures raises the possibility that both are arising from a common precursor cell. Multipotential precursors have been demonstrated *in vivo* during the development of both the retina (Turner and Cepko, 1987; Wetts and Fraser, 1988) and intracortical forebrain, including the neostriata of both mammals (Temple, 1989; Halliday and Cepko, 1992) and birds (Galileo et al., 1990; Gray and Sanes, 1992). In the avian neostriatum, these precursors may remain pluripotential even into adulthood (Goldman et al., 1992a). In contrast, in mammalian *neocortical* ontogeny, committed lines of neuronal and glial SZ precursor cells become established prenatally (Levitt et al., 1981, 1983; Walsh and Cepko, 1988; Luskin et al., 1993), while distinct lines of oligodendrocytic and astrocytic precursors evolve thereafter (LeVine and Goldman, 1988).

Even postnatally, however, some rodent SZ precursor cells remain bipotential for oligodendrocytic and astrocytic production (Levison and Goldman, 1993). Indeed, during late embryonic development, both phenotypically committed and pluripotent precursors may coexist in the telencephalic ventricular zone; a bipotential cortical neuronal–oligodendrocytic precursor is present in the E16 rat forebrain (Williams et al., 1991), as are lineage-restricted neuronal, astrocytic and oligodendrocytic progenitors (Price and Thurlow, 1988; Price et al., 1991). Unfortunately, the present data do not allow us to distinguish whether the precursor cells of the adult human brain are committed to restricted phenotypes, or whether they are pluripotential for neurons and glia. As an additional consideration, the culture environment might determine not only the mitotic activity, but also the lineage commitment of these precursors; their phenotypic potential *in vitro* might differ from that realized *in vivo*. Thus, the ability of these precursors to produce neurons in culture does not necessarily suggest their capacity to do so *in vivo*.

The Natural History of Adult Primate SZ Precursor Cells

Given the retention of neuronal precursor cells in adulthood, the absence of neurogenesis in the mature primate brain (Rakic, 1985; Eckenhoff and Rakic, 1988) is intriguing. Although the adult primate appears to exhibit little if any forebrain neurogenesis *in situ* (Rakic, 1985; Eckenhoff and Rakic, 1988), our data suggest that once removed into culture, residual precursor cells may resume neuronal production and differentiation. Indeed, the adult rhesus monkey has been found to have "hot spots" of mitotic cells in the subependymal layer of the anterior horn of the lateral ventricle (Rakic and Kornack, 1993). This observation suggests that telencephalic SZ precursor populations may remain actively mitotic in the adult primate forebrain, but that these cells may fail to generate neurons *in vivo*. Instead, the ability of their progeny to either survive or differentiate into neurons may be compromised passively, by a lack of some rate-limiting trophic agent (Goldman and Kirschenbaum, 1994), or actively, through the tonic suppression of neurogenesis by either soluble or contact-dependent agents within the local tissue environment.

Indeed, even if the newly generated cells could be rescued *in vivo*, whether by the introduction of survival factors lacking in adult primate brain, or by the active inhibition of tonic suppressive agents, their fate and function could not yet be predicted or dictated. Regarding the SZ cells sampled in this study, their location in the inferior horn of the lateral ventricle, in the anteromedial temporal lobe, suggests that they might contribute neuronal progeny not only to the hippocampal archipallium, but also to the subjacent subiculum, parahippocampal gyrus, and entorhinal cortex, or even the adjacent amygdala and prepyriform cortex. If these daughter neurons were induced to survive *in vivo*, we yet have no way of knowing whether their phenotype would be restricted to a prespecified subset of neurons, or whether they could be recruited into any local circuit having a permissive route and substrate for migration and open synaptic space. In this regard, the population of neuronal precursors for the postnatal rat olfactory bulb, to which we referred previously (Corotto et al., 1993; Luskin, 1993), generates a phenotypically and regionally restricted cohort. Neurons generated from these SZ cells migrate to and insert within the olfactory bulb, but do not appear to contribute to other regional circuits. This suggests that these young neurons are subject either to a predetermined restriction of their fate, or a sharply demarcated distribution of environmental cues limiting their migration. Interestingly, the progenitors from which these rodent neurons arise lay in the anterobasal frontal SZ, a region phylogenetically analogous to the human anteromedial temporal SZ (Farbman, 1991), which corresponds to the SZ segment sampled in our present study.

Nonpermissiveness for Reactive Neurogenesis in vivo

In light of the occurrence of compensatory neurogenesis following brain lesion in lower vertebrates, in-

cluding fish (Anderson and Waxman, 1985), lizards (Font et al., 1991) and birds (Cheng and Zuo, 1993), the primate's apparent lack of such restorative neurogenesis is puzzling (Goldman-Rakic, 1980). *In vivo,* the precursor cells may lack either mitotic stimulation or postmitotic signals permitting neuronal differentiation or survival. Alternatively, these precursors may be tonically inhibited from generating neurons after embryogeny; their ability to generate neurons *in vitro* but not *in vivo* may reflect the suppressive effect on neurogenesis of local, tissue-derived factors. The identity of these agents in the mammalian brain remains problematic. In the adult avian brain, the tonic inhibition of neurogenesis by non-estrogenic ovarian influences (Hidalgo and Goldman, 1993) has opened the possibility that gonadally derived peptides, such as members of the inhibin-β family, might be operative in the restriction of neurogenesis in the adult avian forebrain. Indeed, the close structural homology of ovarian inhibin-β to transforming growth factor β (TGF-β) might suggest a more general role for the latter in the regulation of neurogenesis in adulthood; among olfactory precursor cells, for example, TGF-β has already been shown to potentiate neuroblastic departure from the cell cycle and terminal neuronal differentiation (Mahanthappa and Schwarting, 1993).

Teleologically, it is a reasonable assumption that the nonpermissiveness of the adult brain toward compensatory neurogenesis has adaptive value, particularly in light of the progressive restriction in the temporal and geographic extents of adult neurogenesis as one progresses in phylogeny (Rakic and Kornack, 1993). Nonetheless, whatever the basis for this phylogenetic restriction in adult neurogenesis, our data suggest that it does not stem from a lack of appropriate progenitor cells. Rather, once removed from the milieu of adult brain parenchyma, residual precursor cells can resume the production of antigenically and physiologically characteristic neuronal progeny.

Notes

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RESEARCH/CLINICAL UPDATE

January 7, 2000

RESEARCHERS FIND KEY CELLS IN ADULT BRAIN THAT MAY SOMEDAY REPAIR MYELIN IN MS

Summary:

- Researchers at Cornell University Medical College, supported by the National MS Society, have for the first time isolated cells in the adult human brain that can divide and grow into myelin-making cells and that may ultimately be capable of replacing those damaged in multiple sclerosis.
- Although very basic in nature, this research may eventually lead to therapies for MS either through implantation of such cells, or through development of ways of stimulating progenitor cells resident in a person's brain to produce new oligodendrocytes that can repair myelin damaged by MS and possibly restore nerve function.

Details: National MS Society-supported investigators led by Steven A. Goldman, MD, PhD, of Cornell University Medical College, have reported the discovery and isolation of a population of immature ("progenitor") myelin-making cells (oligodendrocytes) in the brains of adult humans. These cells have the potential to repair myelin that has been destroyed by MS, and possibly to aid in the recovery of function.

Reporting in the November 15 issue of *The Journal of Neuroscience*, the investigators describe having found that the oligodendrocyte progenitor cells are surprisingly abundant in adult brain matter, and are capable of dividing to produce new oligodendrocytes. Most adult human brain cells do not divide. This is the first demonstration that such cells can be stimulated to divide and give rise to new oligodendrocytes.

Background

Throughout the 1990s, researchers had been searching for oligodendrocyte progenitor cells in the human brain. Oligodendrocyte progenitor cells had been found in the rat brain in the 1970s and 1980s. Immature oligodendrocytes had been found in human

brain tissue, but none of these had been capable of dividing. Researchers had begun to conclude that oligodendrocyte progenitor cells capable of dividing did not exist in the adult human brain. But by using surgically removed samples of adult human brain tissue, combined with newly developed techniques of molecular cell identification and separation, Goldman and colleagues were able to refute this notion and for the first time, segregate a population of dividing oligodendrocyte progenitors in adult brain.

The study

In this study, reported in the November 15 issue of *The Journal of Neuroscience*, adult human brain cells were obtained from brain matter that was removed from eight patients ranging in age from 24 to 65 years old, who underwent surgery for a variety of disorders. The investigators used a technique they had developed and tested in animal brain cells to separate living progenitor cells from the larger brain cell population.

The investigators identified a discrete population of oligodendrocyte progenitor cells, which they estimated to represent about four percent of the population of cells in the in the white matter of the brain. They then segregated the progenitor cells, and demonstrated that they were capable of dividing, "more or less on demand," says Goldman.

What the Study Means

This study shows that oligodendrocyte progenitor cells exist within the adult human brain and are capable of dividing. Furthermore, this study describes a method for the isolation and actual purification of these cells, potentially in large numbers. This raises the possibility that patients with MS might someday be treated either by transplanting oligodendrocyte progenitor cells, or by stimulating the patients' own oligodendrocyte progenitor cells to divide and produce new cells. Treatments might also be devised that combine elements of both approaches.

Dr. Goldman's team is currently conducting studies to determine whether transplanted oligodendrocyte progenitor cells will be able to produce replacement myelin on nerve fibers whose myelin has been destroyed. Studies will also be needed to determine whether stimulating the growth of new oligodendrocytes from progenitor cells that exist within a patient's brain will remyelinate damaged neurons.

The National MS Society is actively funding these and other efforts to find ways to repair myelin and nerve cells that have been destroyed by multiple sclerosis, with the hope of restoring nerve function.

From: Research Programs Department

SUMMARY OF MS RESEARCH PROGRESS - 1999

[redacted]

December 10, 1999

This has been another exciting year for MS research. Thanks to funds provided by its chapters and private donors, the National Multiple Sclerosis Society was able to spend a record $22.5 million to support research programs in 1999. Since its founding 53 years ago, the Society has invested more than $260 million to find the cause, treatments and cure for MS.

During the year, our volunteer scientific advisors reviewed 300 MS research proposals and approved 129 as being of high scientific merit and relevance and thus warranting the Society's support. The Society now has over $40 million in current and future commitments to over 300 MS research projects, for which money must be raised.

Significant advances have been made in both laboratory and clinical studies in MS. As the world's largest private supporter of MS research, the Society has been at the core of many of these advances during 1999. Key highlights include:

- In separate Society-supported studies, investigators reported evidence for the possibility that human herpes virus-6 (University of Wisconsin) and the bacterium Chlamydia pneumoniae (Vanderbilt University) may be linked to MS. Further studies are ongoing to determine whether either these or other infectious agents are causal factors in MS, and whether drugs to fight these agents can help MS.

- The first large-scale clinical trial of Copaxone in primary-progressive MS was begun. It will eventually enroll 900 people at 54 centers across the U.S. and Canada.

- Society-supported investigators at Cornell University Medical College reported, for the first time, being able to isolate immature ("progenitor") myelin-making cells in the adult human brain, remove them surgically and transform them, in laboratory dishes, into mature cells capable of making new myelin. This important step may provide a basis for new strategies for repairing damaged myelin in MS.

- The Society launched the Sonya Slifka Longitudinal MS Study. This first study of its kind in the U.S. will collect, on a long-term basis, in-depth information on a national sampling of people with MS in order to address important research questions.

- Society-sponsored researchers at the Mellen MS Center in Cleveland announced results of a study suggesting that individuals with the relapsing-remitting form of MS show progressive loss of brain volume, or atrophy, and that this atrophy may be slowed with interferon beta treatment.

- Investigators at the Weizmann Institute in Israel reported that feeding mice and rats an oral form of Copaxone made their MS-like disease less severe. A large-scale human trial of an oral (pill) form of Copaxone for MS is now being planned. (This drug is currently approved in the U.S. as a daily under-the-skin injection for relapsing-remitting MS.)

- Researchers at Mayo Clinic reported that plasma exchange therapy led to neurologic recovery in about half of 22 people they studied who experienced acute, severe attacks of MS or related disorders and whose neurological deficits were not improved after standard treatment with high-dose steroids.

- As part of the Society's targeted research initiative on gender differences in MS, a small-scale clinical trial of the pregnancy hormone estriol was begun at the University of California at Los Angeles to determine whether it is safe and whether it can control or inhibit MS attacks.

- A small-scale clinical trial of a combination of Avonex and Copaxone in relapsing-remitting MS was begun at 5 centers in the U.S., and an international study was launched to compare the effectiveness of Rebif vs. Avonex in relapsing-remitting MS.

- With Society support, doctors at the University of Southern California began a controlled, Phase 2 clinical trial of "T-cell vaccination" in 80 people with secondary-progressive MS. The "vaccine" is designed to specifically kill immune cells that recognize and launch attacks against myelin insulation in the brain and spinal cord.

- Two experimental treatments were brought before the FDA for approval to treat secondary-progressive MS: Novantrone, a potent immune-suppressing chemotherapy drug; and Betaseron, an immune-system modulator currently approved for relapsing-remitting MS. We should learn in 2000 whether either becomes the first approved treatment for a progressive form of MS.

This fruitful year has brought us closer to achieving our goal: to end the devastating effects of multiple sclerosis.

– Research Programs Department

Beyond the Gray Area

Study finds generating cells in brain's white matter

By Jamie Talan
STAFF WRITER

WHILE RESEARCHERS revel in the recent news that adult brain cells grow and divide in some areas of the gray matter — the brain tissue where neurons do their work — few people have paid attention to the inner workings of the white matter, the brain tissue covering the gray matter.

Dr. Steven Goldman and his colleagues at Weill Cornell Medical College in Manhattan have evidence of young, dividing cells in the white matter in the adult human brain. The hope is to "mass produce" sufficient numbers of these progenitor, or stem cells, for implantation and cell-based therapies to treat multiple sclerosis, stroke, Tay Sachs and other diseases, Goldman said. The study appears in the Journal of Neuroscience.

White matter is a pool of myelin, or insulation, that surrounds the neurons in the gray matter. It is filled with cells that provide nutritional support to the neurons, including oligodendrocytes and androcytes. The classic lesions identified in patients with multiple sclerosis are found in white matter. Also, one-third of stroke victims suffer lesions in this tissue.

Newsday Photo / Patrick Andrade

Dr. Steven Goldman, of the Department of Neurology at Weill Cornell Medical College in Manhattan, studied the brain's white matter.

The researchers have developed a method to isolate these progenitor cells by linking them to a gene for a jellyfish fluorescent protein and using a cell-sorting device to separate them from other types of brain cells.

With this pure population of progenitor cells in hand, Goldman said, it would be possible to develop specific techniques to be used after a brain trauma to replace this insulating material and, in theory, fix the brain's transmission problems. (The myelinated axons are the brain's version of telephone wire, long projections that send signals from cell to cell and enable neurons to communicate.)

Goldman, a neurologist, working with his colleague, Neeta Ray, isolated the newly dividing cells from live tissue taken from epilepsy patients and others undergoing brain surgery or biopsy.

Oligodendrocytes are necessary to make myelin, the insulating sheath that surrounds nerve cells that make communication possible. In multiple sclerosis, oligodendrocytes are inflamed or die as the nerve cells' myelin are shredded.

Astrocytes, support cells that provide metabolic support for neurons, are known to grow and divide, but it was always believed that oligodendrocytes, like neurons, do not re-populate. And while scientists have known that the oligodendrocytes are the cells targeted in MS, the immune system disease has always puzzled researchers because the symptoms — tingling in the extremities, tiredness and loss of normal movement — wax and wane. The white matter lesions seem to get better, as well, and it has never been clear how or why axons re-myelinate.

Goldman, a researcher who started his career studying neurogeneais in canary brains, was convinced that there must be a population of oligodendrocyte-progenitor cells that was helping the adult brain heal itself — at least temporarily.

Working side by side with neurosurgeons, Goldman was able to amass snippets of brain tissue from dozens of patients.

By culturing the tissue, the researchers were able to sort out the oligodendrocytes and watch them grow and divide.

In more recent, unpublished work conducted to

See BRAIN on C6

DID YOU KNOW?

By Kathy Wollard

EMEMBER "You're only as young as you think you are"?

AP Photo, 1984

antelope, at the Cincinnati Zoo in 1984, was transferred as an
l antelope in an effort to preserve the rare species.

lg vs. Extinction

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coming the problems of breeding in captivity, including the obvious fact that in a lab scientists have to deal with the problem of animals who have been removed from their natural surroundings.

George Amato of the Wildlife Conservation Society's Science Resource Center and the Bronx Zoo in New York City, said cloning is one tool that can be used to save rare animals, but he's not enthusiastic about the method in his own research.

Amato is using molecular genetic techniques to help conserve endangered species and, like Dresser, maintains a frozen zoo of sorts — a collection of frozen DNA. Amato said that when rare animals become difficult to observe through traditional methods because they can't be found, DNA helps the scientists in their research.

"We maintain frozen DNA from animals from hundreds of species, most of which are endangered," Amato said. His frozen DNA collection includes the the black rhinoceros from Tanzania, endangered yellow-shouldered Amazon parrots from Venezuela and American crocodiles from Belize.

Despite his reluctance to use cloning, Amato said there is a place for it.

"I do believe it has an application for very specific cases," he said. "The panda is probably a reasonable case. I think that we have some responsibility to the things living we are driving to extinction."

Mind Over Brain's White Matter

BRAIN from C5

test whether the cells are functional, the investigators implanted the tissue containing the oligodendrocytes into animals with a demyelinating disease.

The new tissue developed into oligodendrocytes and made myelin protein, but whether they line up along the axon is still not known, Goldman said.

"It may one day be possible to activate a person's endogenous stem-cell population and generate these cells on demand," Goldman said.

Another option, he added, is to purify these cells and insert them into the white matter of patients. For example, doctors could inject the progenitor-oligodendrocytes into MS plaques or into the stroke lesions to trigger re-myelination.

Goldman believes that the population of stem cells in white matter may be far easier to identify, purify and manipulate than the small number of progenitor cells found in the subventricular zone of the gray matter deep in the brain.

He said there are 10 times as many white matter support cells, also called glia, as neurons in the brain, and that as much as 4 percent of the cells in the white matter may be progenitor cells.

The neurologist also believes that it will be possible to insert a single gene into the progenitor cells, such as the gene that is abnormal in Tay-Sachs.

This would restore the myelin, which is damaged and leads to early death in childhood. Goldman also believes that stroke will also be a good model for this modern treatment.

"The possibility to do these experiments is here, now," Goldman said.

He thinks this material could be in human trials within three years.

"It's one thing to talk about new brain cells. It's another to be able to use them for treatment," Goldman said.

Goldman's group collaborated with Peter Braun and Michel Gravel of McGill University in Montreal, who cloned the oligodendrocyte-specific protein used in the studies to identify the progenitor cells.

The rush to tap into neurogenesis — the growth of brain cells — is fraught with problems.

As it turns out, brain growth isn't always good, and it isn't always normal. A number of labs are beginning to find that growing cells in a test-tube can lead to a population of abnormal cells. In other words, the cells are growing waywardly like cancers and lose their neuronal functional capacity.

Goldman's work bypasses this problem by finding an abundance of progenitor cells rather than helping a few cells grow and multiply.

Sky Watch

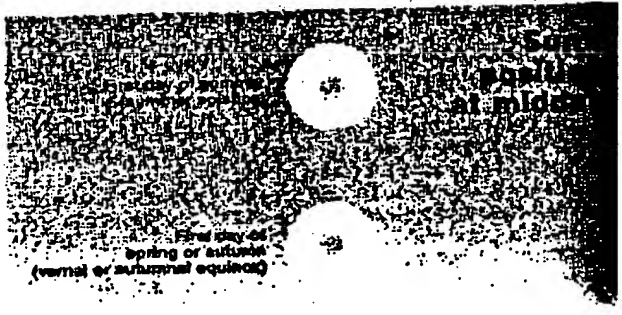
ONE OF MY FAVORITE days of the year is right around the corner. I'm talking about the first day of winter. The reason is the winter solstice, which occurs this year at 2:44 a.m. Dec. 22 on the East Coast. The winter solstice marks the moment that the sun reaches its southernmost position over our planet and begins its journey northward. To an observer on Earth, the day marks the sun's lowest position in the midday sky, and the beginning of its climb once again.

It all happens because our planet's equator is tipped by about 23.5 degrees to the plane of our orbit around the sun. This means that, during this time of year, the Earth's Northern Hemisphere tilts away from the sun, causing the sun's rays to shine down on us at a shallow angle. Six months and half an orbit later, our planet's tilt aims the North-

"solstice" originates in antiquity, coming from two Latin words — "sol" (meaning "sun") and "sistere" (meaning "to stand still").

It is at the winter solstice that the sun's southerly drop seems to end, the sun "stands still," and the star that gives life to planet Earth begins its ascendancy once again. From this moment on, the days become longer, the sun appears higher, and the greening of life gradually return to the Northern Hemisphere of Earth. And not a moment too soon, either!

DENNIS MAMMANA / COPLEY NEWS SERVICE



The Journal of Neuroscience, November 15, 1999, 19(22):9986–9995

Identification, Isolation, and Promoter-Defined Separation of Mitotic Oligodendrocyte Progenitor Cells from the Adult Human Subcortical White Matter

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Previous studies have suggested the persistence of oligodendrocyte progenitor cells in the adult mammalian subcortical white matter. To identify oligodendrocyte progenitors in the adult human subcortical white matter, we transfected dissociates of capsular white matter with plasmid DNA bearing the gene for green fluorescence protein (hGFP), placed under the control of the human early promoter (P2) for the oligodendrocytic protein cyclic nucleotide phosphodiesterase (P/hCNP2). Within 4 d after transfection with P/hCNP2:hGFP, a discrete population of small, bipolar cells were noted to express GFP. These cells were A2B5-positive (A2B5$^+$), incorporated bromodeoxyuridine *in vitro*, and constituted <0.5% of all cells. Using fluorescence-activated cell sorting (FACS), the P/hCNP2-driven GFP$^+$ cells were then isolated and enriched to near-purity. In the weeks after FACS, most P/hCNP2:hGFP-sorted cells matured as morphologically and antigenically characteristic oligodendrocytes. Thus, the human subcortical white matter harbors mitotically competent progenitor cells, which give rise primarily to oligodendrocytes *in vitro*. By using fluorescent transgenes of GFP expressed under the control of an early oligodendrocytic promoter, these oligodendrocyte progenitor cells may be extracted and purified from adult human white matter in sufficient numbers for implantation and cell-based therapy.

Key words: regeneration; myelin; remyelination; cell sorting; stem cells; subependyma

Oligodendrocytes of the adult forebrain are primarily postmitotic. Nonetheless, persistent cycling oligodendrocyte progenitors (OPs) have been described in adult rodent subcortical white matter (Gensert and Goldman, 1996) and may provide a substrate for remyelination after demyelinating injury (Blakemore et al., 1996; Gensert and Goldman, 1997). In humans, the demonstration and identification of persistent subcortical progenitor cells have been more problematic. A pro-oligodendrocytic phenotype has been described in adult human subcortical white matter, although these postmitotic cells may have included mature oligodendrocytes recapitulating their developmental program after dissociation (Armstrong et al., 1992; Gogate et al., 1994). Rare examples of oligodendrocytes derived from mitotic division have been reported in human subcortical dissociates (Scolding et al., 1995), and candidate progenitors have been identified in histological sections on the basis of PDGFα receptor expression (Scolding et al., 1998). Nonetheless, the identification and isolation of viable mitotic oligodendrocyte progenitors from the adult human brain has proven an elusive goal. Indeed, not only have mitotically competent adult human OPs not been preparable in the numbers or purity required for their characterization or functional engraftment, but their very existence in humans has been unclear (Scolding, 1997, 1998).

To establish the existence and relative incidence of oligodendrocyte progenitors in the adult human white matter, we therefore designed a new strategy for the isolation and enrichment of native oligodendrocyte precursors from adult brain tissue. For this purpose, we capitalized on a strategy initially developed for the identification of neuronal precursor cells in which cultured forebrain dissociates were transfected with the gene for green fluorescent protein (hGFP) (Chalfie et al., 1994; Levy et al., 1996), regulated by the early neuronal promoter for Tα1 tubulin (Gloster et al., 1994). This approach permitted the recognition of live, fluorescent neuronal progenitor cells in mixed cell culture. Fluorescence-activated cell sorting (FACS) then permitted the high-yield enrichment and relative purification of these progenitor cells (Goldman et al., 1997; Wang et al., 1998a).

In the present study, we extended this strategy to identify and purify oligodendrocyte progenitors from adult human subcortical white matter. To this end, we used FACS of subcortical cells transfected with hGFP placed under the control of the 5′ regulatory region of an early oligodendrocytic protein, specifically the early promoter (P2) for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; EC 3.1.4.37) (Vogel et al., 1988; Tsukada and Kurihara, 1992). CNP protein is one of the earliest known myelin-associated proteins to be synthesized in developing oligodendrocytes. It is expressed by newly generated cells of oligodendrocytic lineage, even within the ventricular zone, and appears to be expressed by their precursors as well, in both rodents and humans (Scherer et al., 1994; Yu et al., 1994; Grever et al., 1997; Peyron

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Drs. Roy and Wang contributed equally to this work.

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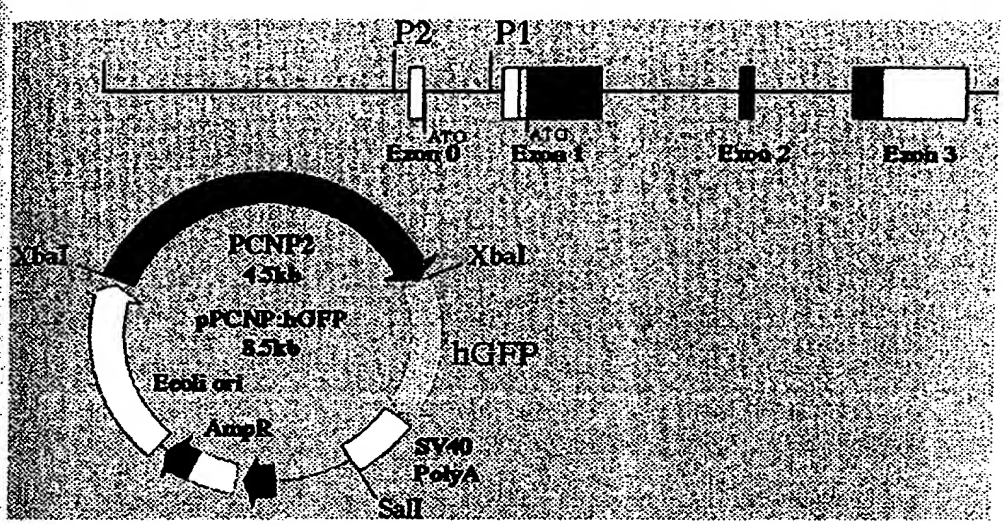

Figure 1. The human P/CNP2:hGFP vector. Humanized GFP (Levy et al., 1996), a mutant form of red-shifted GFP optimized for expression in human cells, was placed under the control of the human CNP2 promoter (P/hCNP2) (Gravel et al., 1996). To construct P/h*CNP2*:hGFP, an *XbaI-XbaI* fragment encompassing exon 0 and part of intron 1 was isolated from the SK/hgCNP plasmid, which contains the complete sequence of the human CNP gene (Gravel et al., 1996), and was then linked to the hGFP gene fused to the SV40 polyadenylation signal.

et al., 1997; Chandross et al., 1999). Importantly, the 5′ regulatory region of the CNP gene includes two distinct promoters, P2 and P1, which are associated with two distinct RNAs, and are sequentially activated at different developmental stages (Douglas et al., 1992; Douglas and Thompson, 1993; Monoh et al., 1993; Scherer et al., 1994) (Fig. 1). Only the CNP mRNA transcribed from the more upstream promoter, P2, is found in the fetal brain, suggesting that the P2 promoter (P/CNP2) directs expression to young oligodendrocytes and their precursors (O'Neill et al., 1997; Gravel et al., 1998). As a result, the CNP2 promoter was chosen for this study for its ability to target transgene expression to oligodendrocyte progenitors and their immature progeny in rodents. On this basis, we postulated that the human homolog of the CNP2 promoter, P/hCNP2, would similarly target transgene expression to human oligodendrocyte progenitor cells.

We report here that the P/hCNP2 has indeed allowed us to direct expression of a reporter gene to oligodendrocyte progenitor cells of the adult human brain and to thereby identify and isolate these cells. Plasmids of hGFP under the control of P/hCNP2, transfected into dissociated subcortical cultures, identified a population of bipolar, primarily A2B5-immunopositive ($A2B5^+$) precursor cells. These cells typically incorporated the mitotic marker bromodeoxyuridine (BrdU) from the culture media and developed oligodendrocytic antigenic expression *in vitro*. Using FACS, we isolated these P/hCNP2:$hGFP^+$ cells from surgically resected subcortical white matter and observed their development into mature, galactocerebroside$^+$ oligodendrocytes in the weeks thereafter. This strategy has allowed us to establish the existence of a distinct class of mitotically competent oligodendrocyte progenitors in the adult human white matter. In addition, P/hCNP2:hGFP-based FACS has enabled us to isolate and separate these cells, viably and in high-yield, and in numbers and purity sufficient to study their cell biology and suitability for engraftment.

Parts of this paper have been published previously in abstract form (Wang et al., 1998b).

MATERIALS AND METHODS

Plasmid construction

*P/hCNP2:hGFP and P/hCNP2:*lacZ. hGFP, a mutant form of GFP optimized for expression in human cells (Levy, 1996), was placed under the control of the human CNP2 promoter (Douglas et al., 1992; Monoh et al., 1993; Gravel et al., 1996). The human CNP gene had been isolated previously (Gravel et al., 1996) by screening a human fibroblast genomic library with a cDNA probe for rat CNP1 (Bernier et al., 1987). The human CNP gene was then subcloned into pBluescript, and the resultant plasmid was designated SK/hgCNP. This plasmid was digested with *Bgl*II and *Xho*I to delete much of the gene downstream of the promoter region. The remaining *Bgl*II and *Xho*I ends were then filled in and blunt-end ligated, yielding plasmid SK/P1P2hCNP, in which both *Bgl*II and *Xho*I were regenerated. A 1123 bp *Xho*I-*Xho*I fragment containing SV40 SD/SA-GFPh-SV40 poly(A^+) was then excised from pTα1:hGFP (Wang et al., 1998) and subcloned into *Xho*I-digested SK/P1P2hCNP to generate the plasmid P/P1P2hCNP:hGFP. The orientation of the hGFP insert was then determined by restriction enzyme mapping.

To construct P/hCNP2:hGFP, the Tα1 tubulin promoter region was excised from pP/Tα1:hGFP using *Xba*I and replaced with the hCNP2 promoter obtained by digesting SK/hgCNP with *Xba*I. The orientation of the P/hCNP2 insert was also determined by restriction enzyme mapping. Similarly, P/hCNP2:*lacZ* was constructed by removing the Tα1 promoter from the Tα1:*lacZ* (Wang et al., 1998a) with *Xba*I, and replacing it with the *XbaI-XbaI* fragment containing the hCNP2 promoter. P/CMV:hGFP was constructed as reported previously (Wang et al., 1998a).

Adult human brain white matter dissociation and culture

Adult human brain tissues, obtained freshly in the course of surgical resection, were collected directly into Ca^{2+}/Mg^{2+}-free HBSS. The white matter was dissected from the rest of the tissue, cut into pieces of ~2 mm on edge, or 8 mm^3, and rinsed twice with PIPES solution (in mM: 120 NaCl, 5 KCl, 25 glucose, and 20 PIPES). It was then digested in prewarmed papain–PIPES solution (11.4 U/ml papain; Worthington, Freehold, NJ) and DNase I (10 U/ml; Sigma, St. Louis, MO), on a rocking shaker for 1 hr at 37°C. The tissue was then collected by centrifuging at 200 × *g* in an IEC Centra-4B centrifuge, resuspended in DMEM–F-12–N2 with DNase I (10 U/ml), and incubated for 15 min at 37°C. The samples were again spun, and their pellets were recovered in 2 ml of DMEM–F-12–N2. They were then dissociated by sequentially triturating for 20, 10, and 5 times, respectively, through three glass Pasteur pipettes fire polished to decreasing bore diameters. Undissociated tissue pieces were eliminated by passage through a fine 40 μm mesh. The cells were collected and rinsed once with DMEM–F-12–N2 containing 20% plasma-derived FBS (PD-FBS; Cocalico Biologicals, Reamstown, PA) to stop the enzymatic dissociation and then resuspended at 1×10^7 cells/ml in DMEM–F-12–N2 containing 10% FBS. The cell suspension was plated at 0.1 ml/dish into 35 mm Falcon Primaria plates coated with laminin (2 μg/cm^2) and incubated at 37°C in 5% CO_2. After 4 hr, an additional 0.7 ml of DMEM–F-12–N2 with 2% PD-FBS was added into each plate. This medium was supplemented with PDGF AA (20 ng/ml; Sigma), FGF-2 (20 ng/ml; Sigma), NT-3 (20 ng/ml; Regeneron Pharmaceuticals, Tarrytown, NY), and BrdU (10 μg/ml). Cultures were transfected after 2–6 d *in vitro* (DIV). After transfection, the

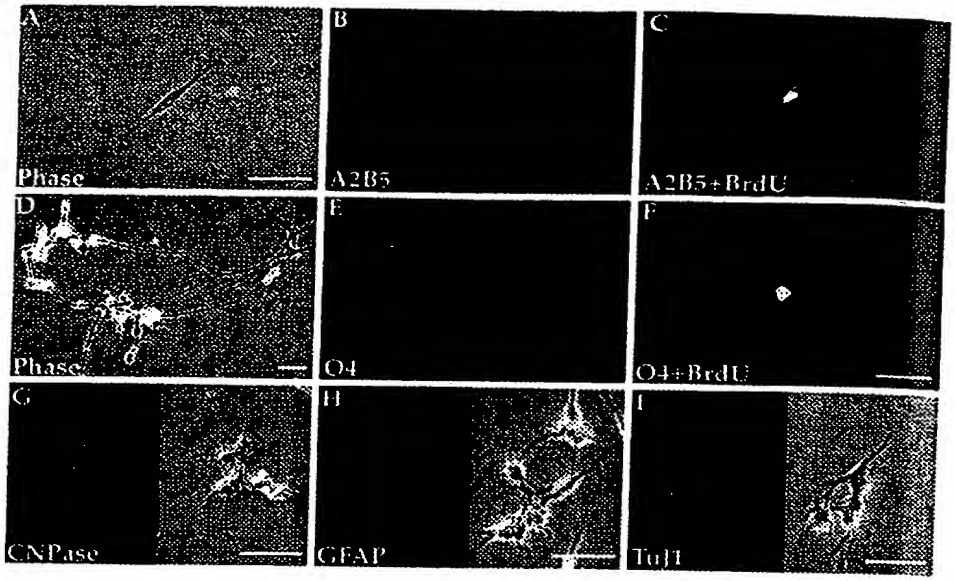

Figure 2. Adult human white matter harbors oligodendrocyte progenitors. Immunocytochemistry of white matter dissociates for a panel of cell type-selective antigens revealed a diverse representation of phenotypes before sorting. *A–C*, A typical bipolar cell, double-labeled for A2B5 (*red*) and BrdU (*yellow*), fixed after 4 DIV. *D–F*, A cluster of postmitotic $O4^+$ cells (*D*, *E*) and an overtly less mature BrdU-incorporating $O4^+/BrdU^+$ cell (*F*). all fixed after 7 DIV. *G–I*, Representative examples of the diverse phenotypes present in the adult white matter. These included cells expressing CNP (*G*), GFAP (*H*), and TuJ1 (*I*) immunoreactivities, which respectively identify oligodendrocytes, astrocytes, and neurons; each cell type was found in the proportion noted in Results. Scale bar, 40 μm.

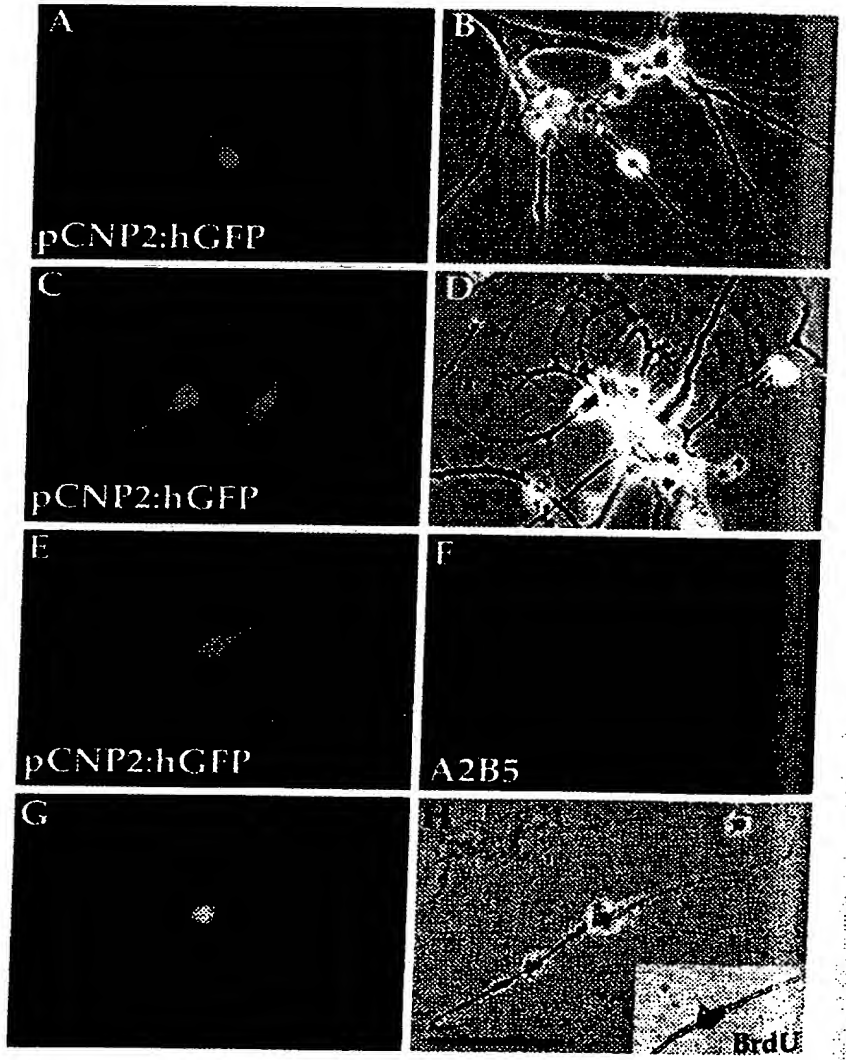

Figure 3. P/hCNP2:hGFP identifies a population of bipolar, $A2B5^+$ cells. GFP expression was observed within 4–5 d after transfection. The P/hCNP2:hGFP$^+$ cells typically first appeared as small, bipolar cells. *A–F*, P/hCNP2:hGFP-expressing cells (*A*, *C*, *E*) and their corresponding phase contrast micrographs (*B*, *D*, *H*). *E*, *F*, Immunocytochemistry identified the P/hCNP2:hGFP$^+$ bipolar cells as $A2B5^+$; *G* indicates double-labeling of the two. *Inset* in *H* shows that this cell incorporated BrdU. Scale bar, 30 μm.

cultures were switched to serum-free DMEM–F-12–N2, with maintained growth factor and BrdU supplementation until FACS.

Transfection

All plasmid constructs were introduced into the cultured cells by liposomal transfection, as described previously (Wang et al., 1998a). Briefly, 2–6 d after plating, each 35 mm dish received a mixture of 2 μg of plasmid DNA and 10 μl of lipofectin in OPTI-MEM (Life Technologies, Gaithersburg, MD). The cells were incubated at 37°C in 5% CO_2–95% air for 6 hr. The transfections were terminated with DMEM–F-12–N2 containing 10% PD-FBS. After 2 hr, the cells were returned to serum free DMEM–F-12–N2 with PDGF AA, NT-3, and FGF2. Imaging for hGFP was first done 2 d after transfection and daily thereafter using an Olympus (Tokyo, Japan) IX70 epifluorescence microscope. The greatest number and proportion of GFP^+ cells were observed 6–7 d after transfection; cultures were therefore sorted at that time point.

Flow cytometry and sorting

Flow cytometry and sorting of $hGFP^+$ cells was performed on a FACS Vantage (Becton Dickinson, Cockeysville, MD). Cells (5×10^6/ml) were analyzed by light forward and right angle (side) scatter and for GFP fluorescence through a 530 ± 15 nm bandpass filter as they traversed the beam of an argon ion laser (488 nm, 100 mW). P/hCNP2:*lacZ*-transfected control cells were used to set the background fluorescence; a false positive rate of 0.02 ± 0.05% was accepted to ensure an adequate yield. For the test samples transfected with P/hCNP2:hGFP, cells having fluorescence higher than background were sorted at 3000 cells/sec. Sorted cells were plated onto laminin-coated 24-well plates, into DMEM–F-12–N2 containing PDGF-AA, NT-3, and FGF2, each at 20 ng/ml. After 4 d, some plates were fixed for immunocytochemistry, and the remainder was switched to DMEM–F-12–N2 containing 10% PD-FBS. After an additional 3 weeks *in vitro*, the sorted cells were stained for either CNP, O4, TuJ1, or glial fibrillary acidic protein (GFAP) immunoreactivities; each was double-stained for BrdU as well.

Data analysis

Experimental end points included the proportion of A2B5-, O4-, CNP-, GFA-, and TuJ1-immunoreactive cells in the total sorted population (all nominally GFP^+ after sorting), as a function of time after FACS. At each sampled time point, the respective proportions of $A2B5^+$, $O4^+$, CNP^+, GFA^+, and βIII-tubulin/$TuJ1^+$ cells were compared with each other and with unsorted controls that were similarly dispersed but replated without sorting (after adjusting their cell densities to those of the post-FACS sorted pool). For each combination of treatment (sorted or unsorted), time point (4 d and 3–4 weeks after FACS), and immunolabel (A2B5, O4, CNP, TuJ1, and GFA), the number of stained and unstained cells were counted in 10 randomly chosen fields, in each of three triplicate cultures.

Immunocytochemistry

Cells were immunostained live for A2B5 or O4 (Bansal et al., 1989), or after fixation with 4% paraformaldehyde, for CNP, TuJ1, GFAP, or BrdU. Selected plates were also stained for CD68 or factor VIII, antigenic markers of microglial and endothelial cells, respectively (Kirschenbaum et al., 1994; Raffi et al., 1995). For A2B5 or O4 immunocytochemistry, plates were washed twice with DMEM–F-12–N2 and then blocked with DMEM–F-12–N2 containing 5% normal goat serum (NGS) for 10 min at 4°C. Monoclonal antibody (mAb) A2B5 (clone 105; American Type Culture Collection, Manassas, VA) was used as an undiluted culture supernatant, and mouse mAb O4 (Boehringer Mannheim, Indianapolis, IN) was used at 1:200. Both were applied in DMEM–F-12–N2 for 30 min at 4°C. The plates were then washed with three changes of cold HBSS containing 1% NGS. The secondary antibody, Texas Red-conjugated goat anti-mouse IgM was used at a dilution of 1:50 for 30 min at 4°C. The cells were then washed and fixed with cold 4% paraformaldehyde for 10 min, washed, mounted in SlowFade, and observed using an Olympus IX70 equipped for epifluorescence. Immunocytochemistry for GFAP and TuJ1 was performed according to described methods (Wang et al., 1998a), as was that for TuJ1 and BrdU (Luskin et al., 1997), CD68 (Kirschenbaum et al., 1994), and factor VIII (Leventhal et al., 1999). Selected cultures were also stained for the more mature oligodendrocyte antigens O1 and galactocerebroside, as described previously (Bansal et al., 1989).

RESULTS

Dissociates of adult human white matter harbored a pool of bipolar, $A2B5^+$ cells

To fully characterize the cell phenotypes resident in adult human white matter, papain dissociates of surgically resected frontal and temporal capsular white matter were obtained from eight patients. These included four males and four females, who ranged from 24 to 65 years old. Three patients had temporal lobe resections for medication refractory epilepsy; two were subjected to decompressive resection during or after extra-axial meningioma removal, two samples were taken during aneurysmal repair, and one was taken from the non-neoplastic approach to a histologically benign ganglioglioma. The monolayer cultures resulting from these white matter dissociations were stained after 5–7 DIV for either of two oligodendrocytic markers, which included the epitopes recognized by the A2B5 and O4 antibodies. Additional, matched cultures were stained after 14 DIV for A2B5, O4, or oligodendrocytic CNP protein, and for either neuronal (βIII-tubulin) or astrocytic (GFAP) target antigens.

In the 14 DIV dissociates of subcortical white matter, 48.2 ± 10.7% of the plated cells expressed the oligodendrocytic epitope recognized by mAb O4 ($n = 3$ patients, with a total of 935 $O4^+$ cells among 2041 scored white matter cells; mean ± SD) (Fig. 2). In matched plates, 49.9 ± 4.9% were immunoreactive for oligodendrocytic CNP protein, and 7.3 ± 3.2% expressed astrocytic GFAP. Double-labeling of selected plates revealed that the $O4^+$ and CNP^+ pools were primarily overlapping, with a small proportion of CNP^+/O4-negative ($O4^-$) cells. In contrast, the GFA^+ cells only rarely colabeled as $O4^+$. A small proportion of $TuJ1^+$ neurons (5.2 ± 2.2%) was also observed, as were factor VIII-immunoreactive endothelial cells (11.7 ± 8.9%) and $CD68^+$ microglia (19.9 ± 5.5%). Through 30 DIV, the proportions of oligodendrocytes and neurons in these cultures remained approximately stable, with 51.3 ± 7.0% $O4^+$ cells and 6.0 ± 2.1% $TuJ1^+$ cells, respectively. In contrast, the proportion of GFA-defined astrocytes in these cultures increased from 7.3 ± 3.2% at 14 DIV to 15.9 ± 1.4% at 30 DIV ($p < 0.01$ by Student's t test).

Notably, a distinct population of small bipolar cells, which expressed A2B5 but which otherwise expressed neither neuronal nor oligodendrocytic phenotypic markers, was observed; these constituted 1.8 ± 0.4% ($n = 5$ patients) of all cultured white matter cells at 7 d. However, these cells became scarcer with time *in vitro* by 30 DIV, and $A2B5^+$ cells constituted <0.1% of the total cultured cell pool.

The CNP2 promoter targeted GFP expression to a bipolar, $A2B5^+$ phenotype

To identify either oligodendrocyte progenitor cells or their immature progeny, white matter dissociates were next transfected after 2–6 d with plasmids encoding P/hCNP2:hGFP. Within 4 d after transfection with P/hCNP2:hGFP, a small proportion of GFP^+ cells were noted. These were invariably small, bipolar cells and constituted <1% of the total cell pool (Fig. 3). After an additional 4–7 d *in vitro*, the cultures were immunostained for one of three oligodendrocyte lineage markers, which included A2B5, O4, and CNP protein, or for either astrocytic GFAP or neuronal βIII-tubulin. At that point, the GFP^+ cells could generally be described as $A2B5^+/O4^\pm/GFAP^-/TuJ1^-$; 62.5 ± 8.8% of $P/hCNP2:hGFP^+$ cells expressed A2B5-IR, 21.1 ± 7.5% were $O4^+$, and another 7.3 ± 3.2% expressed astrocytic GFAP. None were recognized by mAb TuJ1, which targets neuronal βIII-tubulin (Menezes and Luskin, 1994). Thus, within the first 7–10

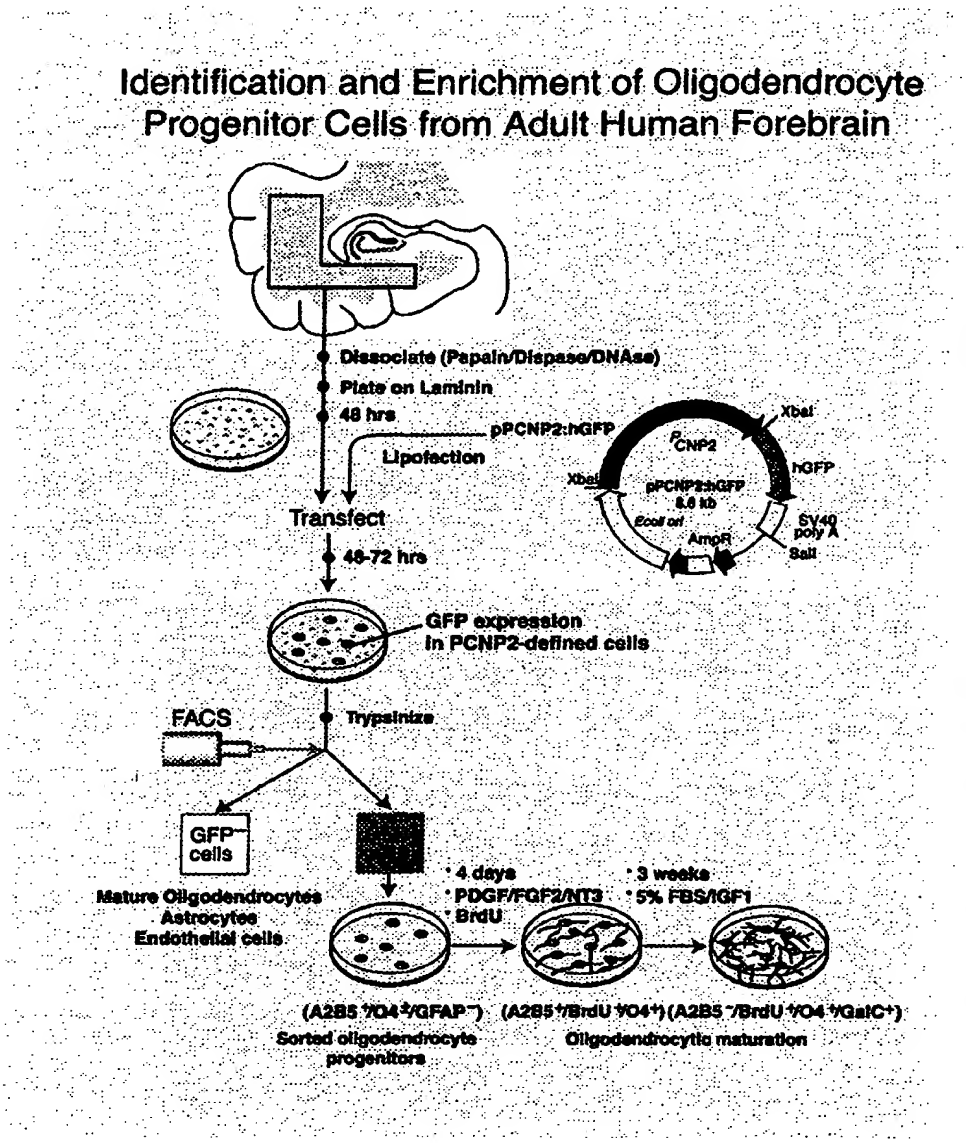

Figure 4. Culture, isolation, and enrichment of oligodendrocyte progenitors. Adult human subcortical white matter, derived from surgical samples of frontal and temporal lobe, was dissected and enzymatically dissociated using papain and DNase and then cultured and transfected with either P/hCNP2:hGFP or control plasmids (P/CMV:hGFP and P/hCNP2:*lacZ*).

d in culture, P/hCNP2:hGFP selectively identified a population of bipolar, A2B5$^+$ cells. When followed over the weeks thereafter, most of these P/hCNP2:hGFP$^+$ cells developed into oligodendrocytes, which could be recognized by their small, multipolar, heavily branching profiles. Indeed, by 4 weeks, most P/hCNP2:hGFP$^+$ cells expressed O4, whereas only rare cells (<1%) continued to express A2B5 immunoreactivity.

P/hCNP2:hGFP-identified cells were mitotic *in vitro*

Among white matter dissociates continuously exposed to BrdU and transfected with pP/hCNP2:hGFP on day 4 *in vitro*, 55 ± 14.8% of the resultant P/hCNP2:hGFP$^+$ cells incorporated BrdU by day 7 (n = 30 plates, derived from three patients) (Fig. 3). Similarly, 43.1 ± 9.1% (n = 5 plates) of the A2B5$^+$ cells in matched plates incorporated BrdU over the same time period. Morphologically, essentially all of these A2B5$^+$ and BrdU$^+$ cells were bipolar at 1 week (Fig. 2). In contrast, the large majority of morphologically mature oligodendrocytes failed to incorporate BrdU *in vitro*. Only 2.1 ± 1.1% of O4$^+$ cells labeled with BrdU to which they were exposed during the first week in culture, and these few O4$^+$ cells may have just arisen from A2B5$^+$ forebears.

P/hCNP2:hGFP-based FACS yielded a distinct pool of bipolar, A2B5$^+$ progenitors

Using sorting criteria intended for cell type purification, the P/hCNP2-driven GFP$^+$ cells were then enriched and cultured separately (Fig. 4). Immediately after FACS, P/hCNP2:hGFP-separated cells primarily expressed A2B5-IR. Furthermore, the majority of these A2B5$^+$ cells were found to have incorporated BrdU from their culture medium before FACS, indicating their mitogenesis *in vitro* (Fig. 5). Within the week after sorting and with concurrent transfer to higher serum media, most of the sorted cells developed O4 expression and lost A2B5-IR.

Notably, P/hCNP2:hGFP-separable cells were not rare. Among seven patients whose white matter dissociates were trans-

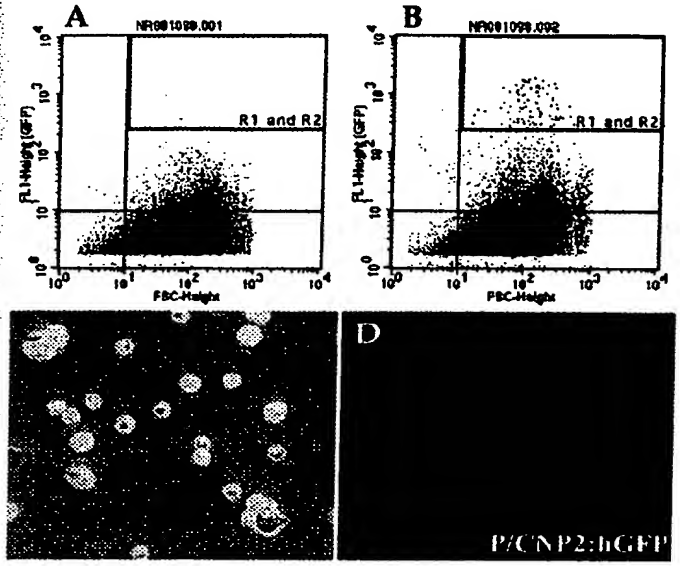

Figure 5. Isolation of P/hCNP2:hGFP$^+$ cells by FACS. *A* and *B* shows a representative sort of a human white matter sample, derived from the frontal lobe of a 42-year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity (FL1), plotted against their forward scatter (FSC, a measure of cell size). *A* indicates the plot obtained from a nonfluorescent P/hCNP2:*lacZ*-transfected control, whereas *B* indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. The boxed area (*R1 and R2*) includes those P/hCNP2:hGFP$^+$ cells recognized and separated on the basis of their fluorescence emission. The many cells thereby recognized in the P/hCNP2:hGFP-transfected sample (*B*) contrasts to the rare cells so identified in the nonfluorescent P/hCNP2:*lacZ*-transfected control (*A*). *C* and *D* show phase and fluorescence images of GFP$^+$ cells 2 hr after sorting. Scale bar, 20 μm.

fected with P/hCNP2:hGFP, 0.59 ± 0.1% of all subcortical cells expressed the transgene and could be separated on that basis. As a result, typically >2000 pCNP2:hGFP$^+$ cells (2382 ± 944) were obtained from sorts that averaged 352,000 gated cells (Fig. 6).

Plasmid transfection favored transgene expression by mitotic targets

The incidence of progenitor cells in the adult white matter may be estimated from the frequency of P/hCNP2-defined cells in these cultures, once the transfection efficiency of this cell population is known relative to the overall white matter cell population. In this regard, our net transfection efficiency, determined using P/CMV:hGFP, was 13.5 ± 2.2% (n = 3 plates; 10 low-power fields of each were scored). This suggested that approximately one cell in eight was successfully transfected with the promoter-driven reporter. On this basis, we estimated that oligodendrocyte progenitor cells might comprise as many as 4% (0.59% × 1/0.135 = 4.37%) of all cells in the subcortical white matter. However, this figure needs to be viewed cautiously, because it assumes that all cells in these cultures were transfected and expressed the plasmid vectors with equal efficiency, regardless of their phenotype or mitotic competence. To test this assumption, we exposed a sample of white matter cultures to BrdU, and 3 d later, transfected them with a plasmid of GFP regulated by the constitutively active cytomegalovirus (CMV) promoter (P/CMV:hGFP) (n = 3 plates, with 15 fields from each scored). One week later, the cultures were fixed, and the relative proportions of mitotic (BrdU$^+$) and postmitotic (BrdU$^-$) GFP$^+$ transfectants were determined.

In keeping with the postmitotic nature of mature oligodendrocytes, only 16.1 ± 1.2% of the cells in unsorted white matter cultures had incorporated BrdU by 10 d *in vitro* (n = 20 fields; mean ± SEM). In these same cultures, 9.4 ± 1.0% of the cells expressed GFP placed under the control of the CMV promoter. Remarkably, however, 78.3 ± 6.5% of these GFP$^+$ cells were BrdU$^+$; this value was over fourfold greater than the BrdU labeling index of the total cell population ($p < 0.01$ by Fisher's exact test). These data suggested that the transduction efficacy of dividing cells in these cultures was substantially higher than that of postmitotic cells. This in turn suggested that mature oligodendrocytes were either transduced with less efficiency or exhibited less efficient transgene expression than mitotically competent oligodendrocyte progenitor cells. As a result, although the P/CNP2 promoter might have been expected to drive transgene expression in oligodendrocytes as well as their progenitor cells, the greater transfection efficiency of dividing cells would have restricted CNP2:hGFP expression to mitotic cells in the oligodendroglial lineage, resulting in selective GFP expression by the oligodendrocyte progenitor pool. Thus, the enhanced transfection and expression of episomal plasmids in dividing cells, combined with the restriction of P/CNP2 transcriptional activation to oligodendrocyte progenitors and their daughters, appeared to collaborate to account for the selective expression of P/CNP2:hGFP by these adult human oligodendrocyte progenitor cells.

P/hCNP2:hGFP$^+$-sorted cells matured primarily, but not exclusively, into oligodendrocytes

Whether mitotic or postmitotic when transfected, the majority of P/hCNP2-sorted cells developed and matured as oligodendrocytes. By 3 weeks after FACS, 74.1 ± 7.7% of these cells expressed oligodendrocytic CNP protein; a matched sample of sorted cells stained after 3 weeks *in vitro* for O4 yielded 66.3 ± 6.8% O4-IR cells, most of which colabeled for the more mature marker galactocerebroside (Fig. 7). Nonetheless, concurrent development of nonoligodendrocytic phenotypes was also noted after FACS purification, albeit at lower frequency than oligodendrocytes; immediately after sorting, 6.5 ± 5.4% of the sorted cells expressed GFAP, and 11.0 ± 4.6% were GFAP$^+$ by 3 weeks *in vitro*. These were not simply false positive contaminants because most were observed to express P/hCNP2:hGFP fluorescence. No P/hCNP2:hGFP$^+$ neurons, as defined by concurrent TuJ1/βIII-tubulin-IR, were observed immediately before FACS. Surprisingly however, 7.5 ± 4.4% of P/hCNP2:hGFP-sorted cells were noted to mature into βIII-tubulin/TuJ1$^+$ neurons in the week after sorting. These TuJ1$^+$ cells were similarly confirmed visually as expressing P/hCNP2:hGFP (Fig. 8). Importantly, the presence of these sporadic P/hCNP2:hGFP$^+$ neurons and astrocytes after FACS suggests that P/hCNP2-defined progenitors may harbor or retain latent multilineage potential, which may be exercised in the low-density, homogeneous cellular environment of the sorted pool.

DISCUSSION

These data indicate that the adult human subcortex harbors a population of residual, mitotically competent oligodendrocyte progenitor cells. The cells constitute a discrete population of bipolar blasts, distinct from mature oligodendrocytes. The progenitors are mitotically competent, and as such, distinct from the much larger population of mature, apparently postmitotic oligodendrocytes. These cells were antigenically immature (A2B5$^+$/O4$^-$) when isolated but matured (O4$^+$/O1$^+$) over several weeks

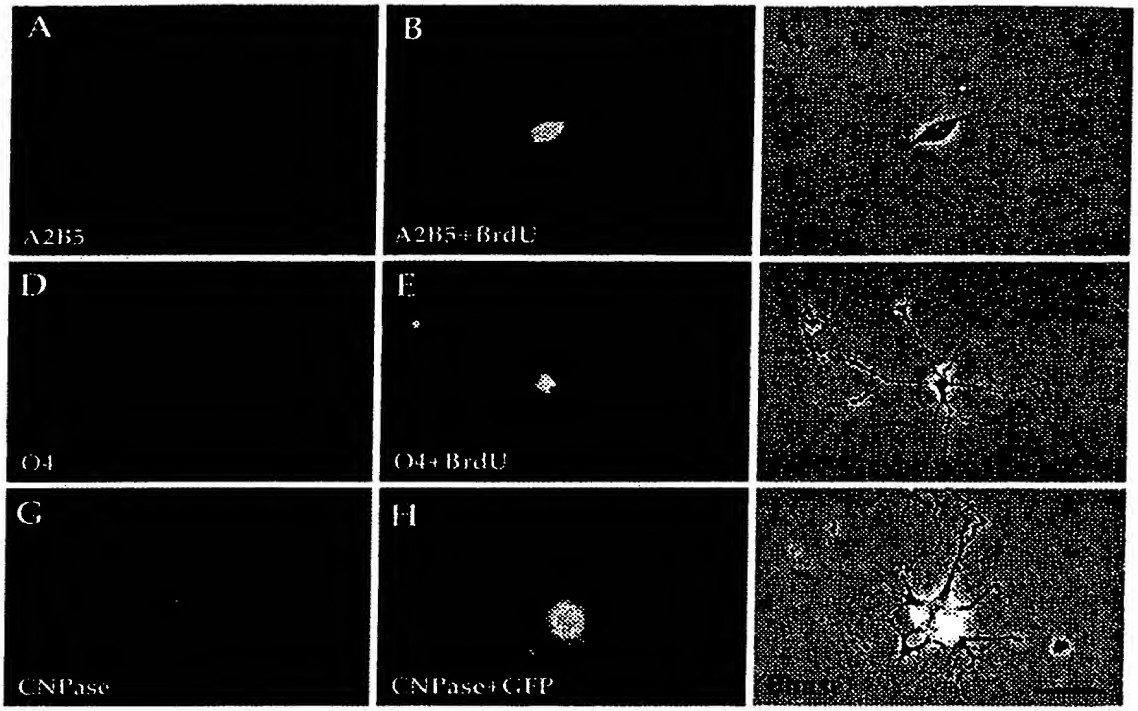

Figure 6. P/hCNP2:hGFP-sorted cells divide and express oligodendrocytic markers. *A–C*, A bipolar A2B5$^+$/BrdU$^+$ cell 48 hr after FACS. *D–F*, Within 3 weeks, the bipolar cells matured into fibrous, O4$^+$ cells. These cells often incorporated BrdU, indicating their *in vitro* origin from replicating A2B5$^+$ cells. *G–I*, A multipolar oligodendrocyte expressing CNP, still expressing GFP 3 weeks after FACS. Scale bar, 20 μm.

in culture. Cell-specific targeted reporting, achieved by transfecting the overall white matter pool with plasmids of GFP placed under the control of the early promoter for oligodendrocytic CNP, allowed the live-cell identification of these progenitor cells. This in turn provided a means for their isolation and purification by fluorescence-activated cell sorting based on P/hCNP2-driven GFP expression.

The nature of the adult white matter progenitor pool

P/hCNP2-defined oligodendrocytic progenitors were not rare. By our sorting criteria, they constituted as many as 4% of cells in the adult human white matter. This figure is marginally greater than previous estimates based on histological identification of PDGFαR expression (Scolding et al., 1998). However, our experiments may have selected for smaller, less fibrous cells, which might be the most capable of surviving tissue dissociation and sorting. Such a bias might have tended to overestimate the incidence of competent progenitor cells in the parenchymal dissociates, so that 4% should be viewed as an upper limit estimate of the incidence of progenitor cells in human subcortical white matter. Nonetheless, the relative abundance and ubiquity of these cells suggest that they may play an important role in the maintenance and function of the normal adult white matter. In rats, a substantial proportion of the white matter cell population is cycling at any one time (Gensert and Goldman, 1996). These cells may be recruited to oligoneogenesis in the event of demyelinating injury (Gensert and Goldman, 1997), and they may be induced to divide *in vitro* by combinations of factors to which they are responsive in development (McMorris and McKinnon, 1996; Shi et al., 1998). Salient differences have been noted in the factor responsiveness of oligodendrocyte progenitors in adult rats and humans, so that the implications of studies on rodent-derived OPs for human oligodendrocyte progenitor biology remain unclear (Scolding, 1998). Nonetheless, the presence of such a large pool of mitotically competent progenitors in humans suggests that some degree of oligodendrocytic turnover may be occurring in the subcortical white matter. This in turn suggests the possible replacement of damaged or dysfunctional postmitotic oligodendrocytes by progenitor-derived recruits. Recent advances in our understanding of both the humoral and contact-mediated control of oligodendrocyte progenitor expansion in rodents (Shi et al., 1998; Wang et al., 1998c) argue that these endogenous progenitors will prove attractive targets for exogenous activation.

Ontogeny and lineage of parenchymal oligodendrocytic precursors

Neural precursor cells are widespread in the subependymal zone of the forebrain ventricular lining (Goldman and Nottebohm, 1983; Lois and Alvarez-Buylla, 1993; Luskin, 1993; Morshead et al., 1994; Kirschenbaum et al., 1994; Kirschenbaum and Goldman, 1995; Pincus et al., 1998) (for review, see Goldman, 1998; Goldman and Luskin, 1998). At least some of these cells may manifest glial antigenicity *in situ* (Doetsch et al., 1999). Whether the P/hCNP2:hGFP-defined subcortical precursors described here are coderived with the subependymal progenitor pool is unknown. It is also unclear whether the P/hCNP2-defined precursors constitute committed oligodendrocyte precursors or whether they are more intrinsically pluripotential and generate given lineages as a function of the environment to which they are exposed. The latter possibility is suggested by the small proportion of P/hCNP2:hGFP$^+$ cells that were found to be GFAP$^+$

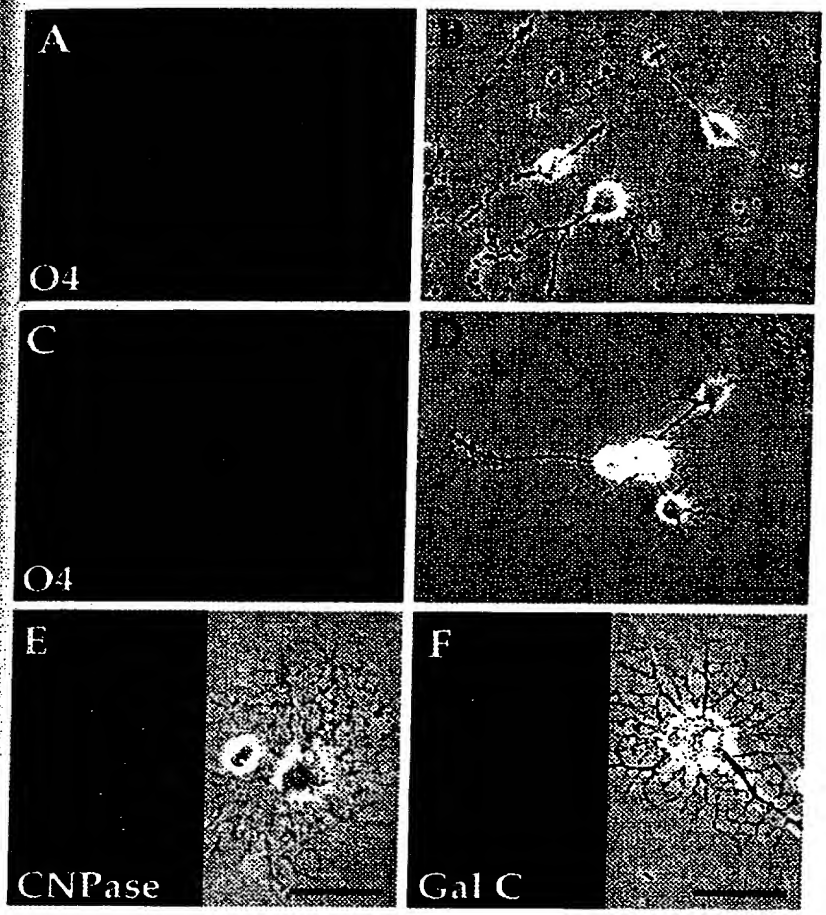

Figure 7. FACS-sorted P/hCNP2:hGFP$^+$ cells mature primarily as oligodendrocytes. *A*, *B*, P/hCNP2:hGFP-sorted cells express O4 (*red*) and begin process elaboration within 4 d after FACS. *C*, *D*, By 2 weeks after FACS, these cells generally develop multipolar morphologies. *Red*, O4-immunoreactive cells. *E*, *F*, Progenitor derived-cells matured further over the following weeks, developing oligodendrocytic morphologies and both CNP protein (*E*) and galactocerebroside (*F*) expression by 4 weeks *in vitro*. Scale bar, 30 μm.

astrocytes upon immunostaining; many of these never developed expression of any oligodendrocytic marker and appeared instead to be astrocytes. This suggests that the P/hCNP2-defined progenitor pool may constitute a bipotential astrocyte–oligodendrocyte progenitor, which may yield primarily oligodendrocytic progeny by virtue of the culture conditions we used. As such, this cell type may well be analogous to its A2B5-defined counterparts in both the perinatal and adult rat optic nerve (Noble et al., 1992; Butt and Ransom, 1993; Colello et al., 1995; Shi et al., 1998).

White matter oligodendrocyte precursors may constitute a pool of multipotential progenitor cells

Whether these cells might also be competent to generate neurons remains unclear. No P/hCNP2:hGFP$^+$ cells were found to ex-

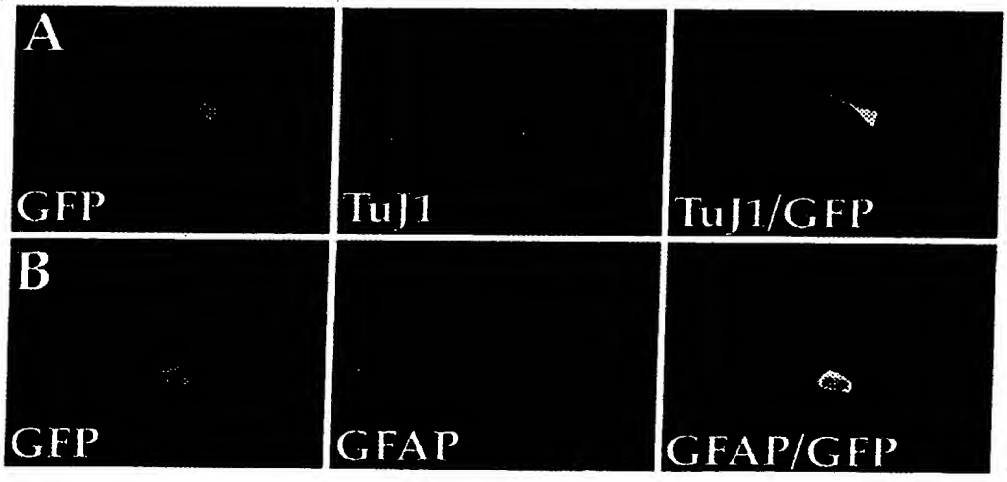

Figure 8. White matter precursor cells may constitute a pool of multipotential progenitors. By 1 week after FACS, some P/hCNP2:hGFP-sorted cells were noted to mature into either TuJ1$^+$ neurons (*A*) or GFAP$^+$ astrocytes (*B*). Both the TuJ1$^+$ (*red* in *A*) and GFAP$^+$ (*red* in *B*) cells were confirmed visually as expressing P/hCNP2:hGFP (*green*). No such neuronal differentiation of CNP2:hGFP-identified cells was ever noted in unsorted plates, within which these cells generally matured as oligodendrocytes and much less so as astrocytes. This suggests that P/hCNP2-defined progenitors may retain some degree of multilineage potential, which may be selectively exercised in the low-density, homogeneous environment of a sorted cell pool in which paracrine influences on differentiation are minimized.

press neuronal TuJ1 in unsorted white matter cultures, of >2000 hGFP$^+$ cells studied. Nonetheless, a small number of TuJ1$^+$ cells were noted to develop in P/hCNP2:hGFP-sorted cultures, and these TuJ1-defined neurons were confirmed as P/hCNP2:hGFP$^+$ and were not nonfluorescent contaminants of the sorts. Thus, with time *in vitro*, particularly in the mitogenic FGF2/PDGF/NT3 environment provided here, it remains possible that these cells retain or regain a capacity for multilineage differentiation, as in development (Williams et al., 1991; Davis and Temple, 1994). Importantly, we only noted P/hCNP2:hGFP-defined cells to mature as neurons after high-grade enrichment by sorting. Thus, the multilineage potential of these cells might be preferentially exercised after their isolation from other cell types in low-density culture. As such, the relative fidelity to oligodendrocytic phenotype by P/hCNP2:hGFP-defined cells in the initial white matter dissociates, before FACS, might reflect an initial restriction of progenitor phenotype by paracrine and/or density-dependent influences *in vitro*. Removal and sorting of these cells to low-density, phenotypically homogeneous culture might effectively remove such paracrine restrictions, in essence revealing a multipotential progenitor cell in the adult subcortical parenchyma.

Implantation for the treatment of demyelinating diseases

The high-yield acquisition of oligodendrocyte progenitor cells from the adult human white matter may allow us to better define those growth and differentiation requirements specific to these cells. The potential use of these cells as substrates for induced remyelination, whether upon endogenous activation or engraftment, suggests therapeutic strategies appropriate to a variety of white matter diseases. These potential therapeutic targets include ischemic demyelination, as in subcortical lacunar infarction and hypertensive leukoencephalopathy, postinflammatory demyelinations, such as radiation necrosis and remitted multiple sclerosis, as well as the degenerative and metabolic leukodystrophies.

Together, these observations suggest that a phenotypically distinct pool of oligodendrocyte progenitor cells persists in relative abundance in the adult human white matter. P/hCNP2:hGFP-based FACS permits their viable harvest in sufficient numbers and purity to enable their potential use in cell-based therapeutic strategies.

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Progenitor Cells Derived From the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes Within Demyelinated Lesions of the Rat Brain

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A distinct population of white matter progenitor cells (WMPCs), competent but not committed to generate oligodendrocytes, remains ubiquitous in the adult human subcortical white matter. These cells are present in both sexes and into senescence and may constitute as much as 4% of the cells of adult human capsular white matter. Transduction of adult human white matter dissociates with plasmids bearing early oligodendrocytic promoters driving fluorescent reporters permits the separation of these cells at high yield and purity, as does separation based on their expression of A2B5 immunoreactivity. Isolates of these cells survive xenograft to lysolecithin-demyelinated brain and migrate rapidly to infiltrate these lesions, without extending into normal white matter. Within several weeks, implanted progenitors mature as oligodendrocytes, and develop myelin-associated antigens. Lentiviral tagging with green fluorescent protein confirmed that A2B5-sorted progenitors develop myelin basic protein expression within regions of demyelination and that they fail to migrate when implanted into normal brain. Adult human white matter progenitor cells can thus disperse widely through regions of experimental demyelination and are able to differentiate as myelinating oligodendrocytes. This being the case, they may constitute appropriate vectors for cell-based remyelination strategies.

Key words: transplant; remyelination; myelin; precursor cells; neural stem cells; cell sorting

Glial progenitor cells biased toward oligodendrocytic production persist within the adult human white matter and may be isolated and propagated as such (for review see Goldman, 2001). These cells appeared to correspond, in both function and phenotype, to analogous progenitors in the adult rodent white matter, whose phenotypic potential had been previously established in vitro (Wolswijk and Noble, 1989) and validated by retroviral lineage analysis in vivo (Gensert and Goldman, 1996, 1997; Levison and Goldman, 1999). The existence of postmitotic pro-oligodendrocytes had previously been determined and characterized in the early 1990s by Dubois-Dalq and her colleagues in a series of elegant studies (Armstrong et al., 1992; Gogate et al., 1994), and these cells were subsequently identified and mapped histologically (Scolding et al., 1998). However, mitotically competent progenitors capable of giving rise to oligodendrocytes were not isolated from adult human brain tissue until later (Roy et al., 1999), when promoter-based sorting permitted the isolation of rare or otherwise hard-to-distinguish progenitor cells from native tissues. In this approach, fluorescent reporters such as green fluorescent protein (GFP) are placed under the control of promoters for genes selectively expressed in the progenitor cells of interest (Wang et al., 1998). The chimeric promoter-driven GFP transgenes are then either transfected or infected into the cell population containing the target progenitor cell, and, upon GFP expression by the cells of interest, the progenitor pool is then extracted by fluorescence-activated cell sorting (FACS). This approach has allowed the identification and isolation of rare neural progenitor cell populations from the ventricular zone and hippocampus, from both fetal and

The first two authors contributed equally to this study.

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adult human samples (Wang et al., 1998, 2000; Roy et al., 2000a,b; Keyoung et al., 2001).

To determine whether adult human white matter might harbor oligodendrocyte progenitor cells, we constructed plasmid vectors containing the early promoter for the oligodendrocyte protein cyclic nucleotide phosphodiesterase, placed 5′ to the coding region for human GFP (hGFP). When we transfected this construct into dissociates of the adult human capsular white matter, we observed that P/CNP2:hGFP was expressed initially by only a single, morphologically and antigenically discrete class of bipolar cells (Roy et al., 1999). These cells were mitotically competent, in that they incorporated bromodeoxyuridine (BrdU) and continued to proliferate in low-serum base medium containing fibroblast growth factor-2 (FGF2), platelet-derived growth factor (PDGF), and neurotrophin-3 (NT-3). Among the P/CNP2:hGFP$^+$ cells, most initially expressed the early oligodendrocytic marker A2B5 but failed to express the more differentiated markers O4, O1, or galactocerebroside. Some expressed astrocytic glial fibrillary acidic protein (GFAP), but none expressed neuronal markers when identified by their GFP fluorescence in mixed, unsorted cultures. When FACS was used to purify these P/CNP2:hGFP$^+$ cells, most were found to mature as oligodendrocytes, progressing through a stereotypic sequence of A2B5, O4, O1, and galactocerebroside expression (Roy et al., 1999), as during development (Noble, 1997). However, occasional neurons were also noted to arise from these cells, particularly in low-density preparations following high-purity FACS, a condition under which sorted progenitors are largely devoid of autocrine and paracrine growth factors. Thus, the nominally glial progenitor of the adult white matter might actually represent a multipotential neural progenitor cell, restricted to the glial and oligodendrocytic lineage by the local white matter environment. As a result, we have designated these cells *white matter progenitor cells* (WMPCs), rather than simply glial progenitors, in recognition of their intrinsically broad lineage potential. Importantly, these cells are not rare: By cytometry based on P/CNP2-driven GFP, WMPCs made up over 0.4% of the sorted white matter cell pool (Roy et al., 1999). With correction for an average plasmid transfection efficiency of 13%, over 3% of dissociated white matter cells might be competent to serve as progenitor cells.

In the present study, we sought to assess the engraftability and myelinogenic competence of these cells when introduced into a region of central demyelination. To this end, we employed a lysolecithin model, in which we injected human WMPCs into the lesioned adult rat brain and then assessed the integration, phenotypic maturation, and myelinogenic competence of the implanted human cells in the environment of the lesioned adult rodent white matter. In addition, we sought to achieve a higher yield means of separating these progenitor cells from surgical samples of the adult white matter, to increase the feasibility of using them in experimental transplantation.

MATERIALS AND METHODS

Adult Human White Matter Dissociation and Culture

Surgically resected adult human brain tissue samples were obtained from five patients ranging from 22 to 49 years old (two males with aneurysms, a female with a geographically distant and histologically circumscribed hemangioma, a female with temporal lobe epilepsy, and a male with an arteriovenous malformation). Surgical resections of forebrain white matter were collected in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS), minced, rinsed twice in PIPES (in mM: 120 NaCl, 5 KCl, 25 glucose, and 20 PIPES), and digested in papain-PIPES (11.4 U/ml papain; Worthington, Freehold, NJ) and DNase I (10 U/ml; Sigma, St. Louis, MO) on a rocker at 37°C for 1.5 hr. The cells were collected by centrifugation at 200*g* in an IEC Centra-4B centrifuge, resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12/N2 with DNase I (10 U/ml), and incubated at 37°C for 30 min. The samples were spun again and the pellets recovered in 2 ml of DMEM/F12/N2. The cells were then dissociated by sequentially triturating for 20, 10, and 5 times, respectively, through Pasteur pipettes fire polished to decreasing bore diameters. Undissociated pieces were removed by passage through a 40 μm mesh. The cells were collected and rinsed with DMEM/F-12/N2 containing 10% plasma-derived fetal bovine serum (PD-FBS; Cocalico, Reamstown, PA) to stop the dissociation. The cells were then suspended in DMEM/F12/N2 containing PDGF-AA (20 ng/ml; Sigma), FGF-2 (10 ng/ml; Sigma), and NT-3 (2 ng/ml; Regeneron, Tarrytown, NY) and plated in 100 mm uncoated petri dishes (Corning, Corning, NY).

Magnetic Separation of A2B5$^+$ Cells

After 48 hr in culture, cells dissociated from adult human white matter were collected by washing the plates with Ca^{2+}/Mg^{2+}-free HBSS. The total number of viable cells was determined using calcein (Molecular Probes, Eugene, OR). The cells were incubated with supernatant of hybridoma cells expressing the monoclonal IgM antibody A2B5 (clone 105; American Type Culture Collection, Manassas, VA). Incubation proceeded for 30–45 min at 4°C on a shaker. The cells were washed three times with 10 times the labeling volume in phosphate buffer containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The cells were incubated with 1:4 microbead-tagged rat anti-mouse IgM (Miltenyi Biotech) for 30 min at 4°C on a shaker. For flow cytometric analysis, some cells were incubated for the same duration with fluorescein isothiocyanate (FITC)-tagged goat anti-mouse IgM (1:50). The cells were washed three times and resuspended in an appropriate volume of buffer. The A2B5$^+$ cells were separated using positive selection columns, type MS$^+$/RS$^+$ or LS$^+$/VS$^+$ (MACS; Miltenyi Biotech).

Labeling of Human Donor Progenitor Cells

Lipophilic dye tagging. Some A2B5-sorted cells were tagged with 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) at 0.01 mg/ml. DiI-tagged cells were noted to retain their fluorescence discretely for at least 1 week after tagging and transplantation. For longer survival times, cells were identified as donor derived and imaged on the basis of anti-human nuclear antibody immunostaining or by BrdU tagging in vitro prior to implantation, with

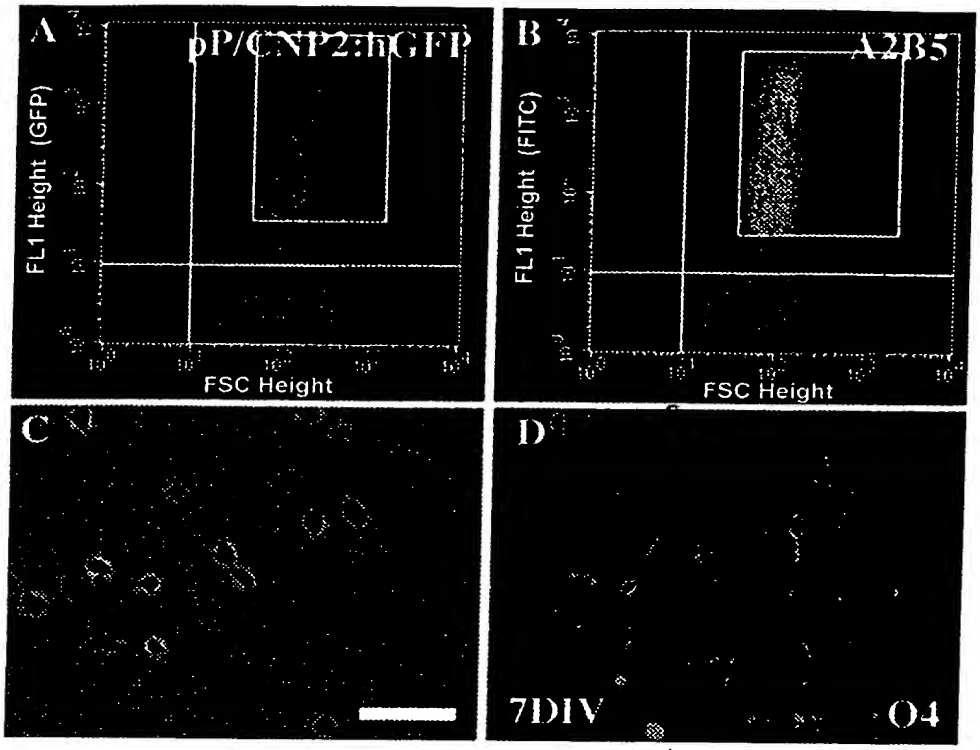

Fig. 1. Oligodendrocyte progenitor cells may be extracted from the adult human white matter. **A,B:** Separation plots of progenitor cells derived from dissociates of adult human white matter. These graphs plot forward scatter (FCS), an index of cell size, against fluorescence intensity (FL1). A shows separation of progenitors based on CNP2:hGFP, and B exhibits FACS separation based on A2B5-immunoreactivity. The two cell populations sort into homologous quadrants on forward scatter, indicating that the two methods extract cells of the same size. They also sort into identical pools as defined by side scatter (not shown), indicating that their shape and internal reflectance are analogous as well. **C,D:** By 7 days in culture, most of the A2B5-sorted population had become oligodendrocytes (C, phase; D, fluorescence). This was manifested in their expression of O4 immunoreactivity (green), which recognizes a sulfatide epitope characteristic of oligodendrocytes. Scale bar = 40 μm.

subsequent BrdU immunodetection. Alternatively, some human donor progenitors were genetically tagged with GFP, using lentiviral delivery.

Lentiviral GFP. In some experiments, the A2B5-sorted cells were infected 24 hr after separation with a purified VSV-pseudotyped lentivirus (1×10^5/ml) constructed to express enhanced GFP (EGFP) under the control of the cytomegalovirus (CMV) promoter, with a WPRE 5 woodchuck posttranscription regulatory element. The latter acts like poly-A by stabilizing the transcript without stopping transcription. The lentivirus was generated by cotransfecting plasmids pCMV-D-R8.91, pMD.G, and pHRCMVGFPwsin into 293T cells (Han et al., 1999). Viral particles were collected after 72 hr. The viruses were partially purified by centrifuging the collected supernatant at 60,000*g* for 2 hr. The sorted A2B5 cells were infected with the virus in the presence of polybrene (8 μg/ml). GFP expression was typically observed by over half of the cells within each infected cell culture within 24 hr after infection. The cells were harvested for transplantation 48 hr after viral infection.

Surgery

Lysolecithin injection. Lesions were produced in the corpus callosa of 200–225 g male rats by stereotaxic bilateral injection of 1 μl of either 1.5% (used for xenografts of GFP-tagged cells only) or 2% lysolecithin type V (Sigma). The coordinates were 1.1 mm posterior to Bregma, 1.0 mm lateral to the midline, and 2.8 mm ventral (Gensert and Goldman, 1997), and the myelinotoxin was delivered at 20 μl/hr.

Transplantation. Three days after lysolecithin instillation, 100,000 A2B5-sorted cells were delivered in 2 μl HBSS into the site of lysolecithin injection, by infusion over 3 min (20 μl/hr). Control animals received comparable injections of saline. Animals were sacrificed at 1 (n = 2), 2 (n = 2), 3 (n = 3), 4 (n = 3), and 8 (n = 1) weeks after xenograft.

Immunosuppression

All animals were immunosuppressed with cyclosporin (Sandimmune; Novartis; 50 mg/ml). Animals received 15 mg/kg daily, beginning on the day of lysolecithin lesion and proceeding daily thereafter until sacrifice. Animals receiving GFP-tagged xenografts were given 20 mg/kg.

Immunohistochemistry

Animals were perfused via intracardiac catheter with HBSS with Ca^{2+}/Mg^{2+}, followed by 4% paraformaldehyde, with postfixation for several hours in 4% paraformaldehyde, passage through increasing concentrations of sucrose to 30%, and freezing during embedding in OCT (Lipshaw). The brains were then cut in 15 μm sections on a Hacker cryostat. Sections were processed for one or more of the following antigenic markers: anti-human nuclear protein (Chemicon, Temecula, CA; MAb1281; 1:50 for 2 days, 4°C), anti-CNP (Sternberger, Baltimore, MD; MAb SMI-91; 1:1,000 overnight, 4°C), anti-human GFAP (SMI 21; 1:1,000, overnight, 4°C), or antimyelin basic protein (anti-MBP; Chemicon Ab980; 1:100 overnight, 4°C). Secondary antibodies included FITC, Cy5, and Texas red-tagged anti-mouse IgM and IgG, anti-rabbit IgG (Jackson

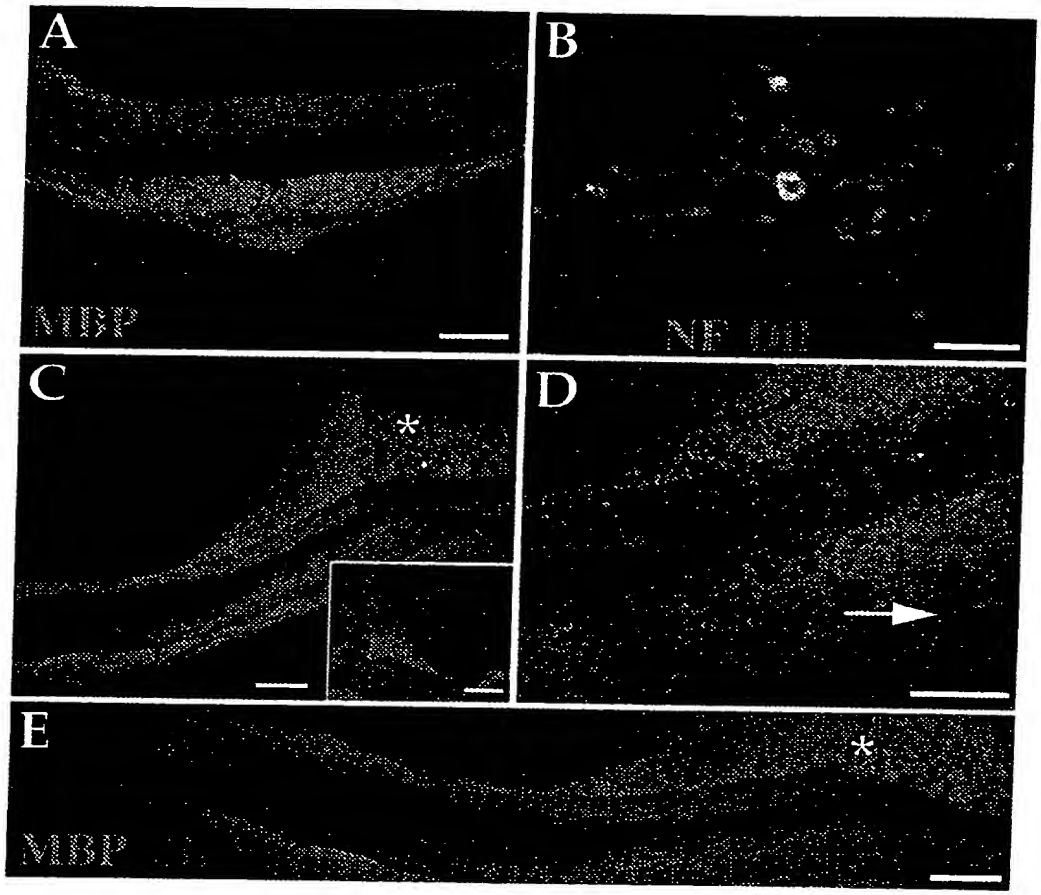

Fig. 2. Implanted white matter progenitors migrated widely throughout the demyelinated callosum. **A–E:** Sorted adult human white matter progenitors were transplanted into lysolecithin-induced demyelinated lesions in the corpus callosa of adult rats. A shows that lysolecithin infusion yielded demyelinated plaques in the subcortical white matter. This rat was injected with 1 μl of 2% lysolecithin-V, directly into the central core of the corpus callosum, then sacrificed for histology 1 week later and immunostained for myelin basic protein (MBP). The large central lesion is visible as the discoid region of MBP immunonegativity, surrounded by the otherwise MBP^+ callosum (green). B: $Neurofilament^+$ axons (green) initially survived lysolecithin lesion, as seen here 1 week after lesion of the callosum. MBP immunoreactivity (green) has been lost from this lesion core, and implanted progenitors have just immigrated to the lesion (orange). However, axonal spheroids were frequent within the lysolecithin lesion bed, indicating some degree of early injury and transection, to which spheroid formation is a response. The ability of implanted progenitors to effect repair is thus limited by the viability and integrity of the targeted axonal cohort. C: DiI-labeled human progenitor cells (red) 1 week after implant. Even at this early time point, the cells extend throughout the demyelinated lesion, which is characterized by its lack of MBP immunoreactivity (green). The cannula track (*) indicates the site of cell injection into the demyelinated lesion, which was induced 3 days before 10^5 sorted, DiI-tagged (red) human progenitors were delivered in 2 μl. **Inset:** Fluorescent microbeads (red) injected into regions of lysolecithin demyelination (MBP; green) failed to disperse beyond their site of injection. D: The transplanted cells migrated throughout the demyelinated plaque, but not beyond its borders, except for occasional migrants that followed the parenchymal surfaces of blood vessels (arrow). The restriction of migration to demyelinated regions suggests that normal myelin impeded the migration of these cells (bottom). E: This low-power montage illustrates the extent and rapidity of migration by engrafted white matter progenitors. Within 1 week of implantation into this demyelinated callosum, the cells traversed the midline to infiltrate the lesion bed in the contralateral hemisphere. The longitudinal extent of this lesion is approximately 6 mm, and the rat was sacrificed 1 week after implantation. Scale bars = 200 μm in A,E; 20 μm in B; 100 μm in C,D; 500 μm in inset.

Immunoresearch, West Grove, PA), and Alexa 488- and 594-tagged anti-mouse and anti-rabbit IgG (Molecular Probes).

Imaging

Brain sections were photographed using an Olympus Fluoview confocal coupled to an IX70 photomicroscope. Images were acquired in both red and green emission channels using an argon-krypton laser, as previously described (Benraiss et al., 2001). The images were then viewed as stacked z-dimension images, both as series of single 0.5 μm optical sections and as merged images thereof. The z-dimension reconstructions were all observed in profile; every human cell doubly labeled with a

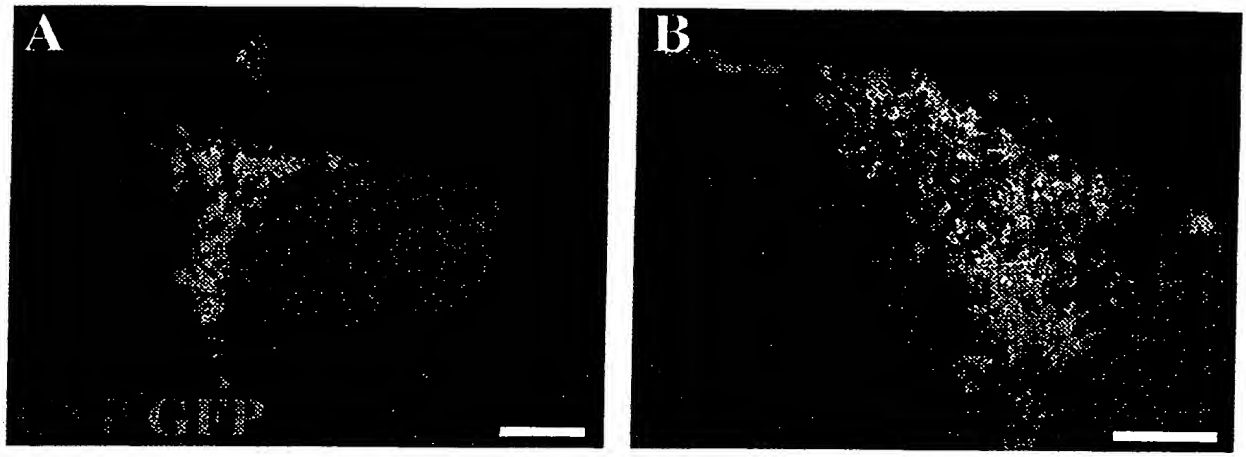

Fig. 3. Normal white matter was nonpermissive for the migration of adult progenitors. A2B5-sorted human progenitors xenografted into the normal adult rat brain failed to migrate beyond the injection site. For this figure, the human cells were prelabeled in vitro with lentiviral GFP (green) to allow the implanted progenitors and their progeny to be identified. **A,B:** The implanted WMPCs shown in two different fields here were imaged in an animal killed 4 weeks after implantation. The implanted cells did not migrate beyond the borders of the initial injectate. This contrasted with the widespread migration of these cells throughout demyelinated foci, as in Figure 2. Scale bars = 200 μm in A; 100 μm in B.

phenotypic marker was observed orthogonally in both the vertical and the horizontal planes to ensure double labeling.

RESULTS

Oligodendrocyte Progenitors of the Human White Matter Are Selected by A2B5 Expression

In previous studies, we found that transduction of adult human white matter dissociates with plasmids bearing the early oligodendrocytic promoter P/CNP2, driving the fluorescent reporter hGFP, permitted FACS of oligodendrocyte progenitor cells from cultured brain tissue (Roy et al., 1999). Because P/CNP2:hGFP$^+$ cells typically expressed A2B5 immunoreactivity, we asked whether separation based on A2B5 might yield the same pool of mitotic oligodendrocyte progenitor cells. To this end, we first used FACS based on A2B5 expression to extract A2B5$^+$ cells from adult WM dissociates (Fig. 1). We found that 2.7% ± 0.4% of the cells could be separated as A2B5$^+$ (n = 5 patients). This compared with P/CNP2:hGFP-based FACS, from which 0.59% ± 0.1% of the cells could be sorted as P/CNP2:hGFP$^+$; the mean transfection efficiency of 13.5% would have predicted that as many as 4.4% of sorted WM cells were potentially P/CNP2:hGFP$^+$. Using that figure as an arbitrary benchmark, we can estimate that A2B5-based FACS achieved the viable extraction of 57.4% (=2.7/4.4 × 100) of the P/CNP2:hGFP-predicted progenitor cells in the adult white matter.

We next used, based on this figure, immunomagnetic sorting (IMS) to select A2B5$^+$ cells from adult WM dissociates. IMS permits a higher yield than FACS, with a greater recovery and higher viability achieved at the expense of a higher incidence of false positives. We found that, by IMS, 2.87% ± 0.7% of the cells were separated as A2B5$^+$ (n = 3 patients). This was in accordance with the incidence of WMPCs estimated by both P/CNP2:GFP-based FACS and A2B5-based FACS.

As with the P/CNP2:hGFP$^+$ cells, the A2B5$^+$ cells were mitotic and gave rise largely to oligodendrocytes (Fig. 2). When exposed to BrdU for the first 2 days after sorting, A2B5-defined cells incorporated the label and expanded in number, indicating their persistent replication in vitro. Over the week thereafter, most began to express definitive markers of the oligodendrocytic phenotype; by 1 week after isolation, >70% expressed the oligodendrocytic antigen O4. Together, these data indicate that A2B5-based FACS and IMS of the adult human white matter yields a population of oligodendrocyte progenitor cells that may be homologous to that recognized by P/CNP2:GFP-based isolation and FACS.

Lysolecithin Lesions Provide Demyelinated Foci Appropriate for Experimental Implantation

To establish whether adult human WMPCs could survive xenograft to adult brain parenchyma, we implanted human WMPCs into lysolecithin-demyelinated callosal lesions in adult rats. Lysolecithin is a useful agent for achieving predictable, focal lesions of the white matter (Gensert and Goldman, 1997). It results in local demyelination with local oligodendrocytic loss, some axonal loss, and relative preservation of astrocytic and endothelial elements. Spontaneous remyelination may occur following lysolecithin lesioning and follows a time course that is dependent on the type of lysolecithin (Sedal et al., 1992), its volume and concentration (Woodruff and Franklin, 1999), and the age of the animals (Shields et al., 1999).

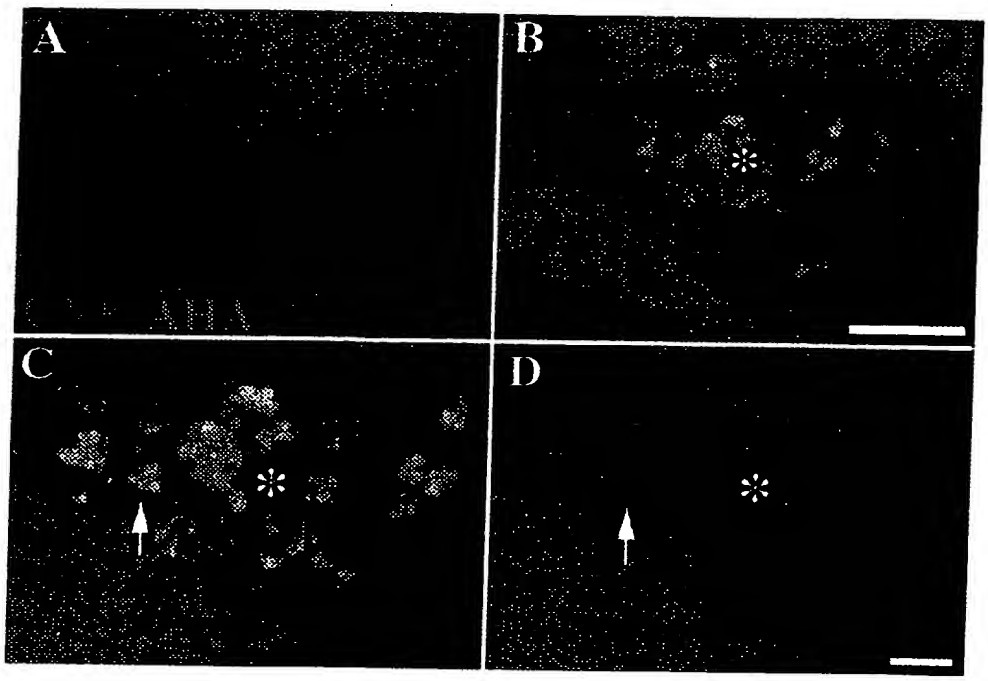

Fig. 4. Xenografted adult white matter progenitors become oligodendrocytes and astrocytes. Implanted A2B5-sorted progenitors typically developed expression of oligodendrocytic CNP protein within 2 weeks of implantation. **A:** In a control animal that received a saline injection, CNP protein (red) remains absent from the demyelinated central core of the callosum (black) 15 days after lesion. In the matched animal shown in **B**, human white matter progenitor cells, recognized by anti-human nuclear antibody (AHA; green), were noted both to fill in the lesion and to express oligodendrocytic CNP (red) 15 days after implantation. **C** shows a higher magnification of this field; **D** demonstrates CNP expression (red) associated with the cell bodies of the human cells visualized as AHA^+ in B. Asterisks are located in the same position; arrows in C and D indicate the same cell. Scale bar = 20 μm in B (for A,B); 5 μm in D (for C,D).

Therefore, lysolecithin lesions mimic salient aspects of acute inflammatory demyelination.

We first confirmed the prior observations of Gensert and Goldman (1997) that lysolecithin lesion was associated with a focal lesion of capsular myelin, with initial injury predominantly limited to oligodendrocytes and their myelin. When assessed 1 and 3 weeks after 1 μl injections of 2% lysolecithin-V, these lesions exhibited a mild degree of reactive astrocytosis within the demyelinated focus, the vascular architecture of which appeared intact. No myelin could be visualized by staining for MBP within 2 mm of the callosal injection site. In addition, oligodendrocytes were markedly diminished, with a >95% loss of CNP^+ cells within the MBP-demarcated lesion (Fig. 2A). Axons were present, as assessed by neurofilament staining, but axonal spheroids were common, indicating some degree of axonal damage and early loss (Fig. 2B).

Adult Human-Derived A2B5-Defined Progenitors Survive and Rapidly Migrate Upon Xenograft to Lysolecithin-Demyelinated Foci of the Adult Rat Brain

We next prepared A2B5-sorted progenitor cell pools from adult human white matter and stereotaxically implanted them into both normal and lysolecithin-lesioned adult rat brain. $A2B5^+$ cells (1×10^5) were implanted into each lesion bed 3 days after a 1 μl injection of 2% lysolecithin. Some donor cells were prelabeled with the lipophilic tracking dye PKH26 to allow their detection after implantation (Horan and Slezak, 1989). Other donor cells were instead localized using human-specific donor cell antigens. At 1, 2, 3, 4, and 8 weeks after implantation, the recipient brains were fixed and prepared for histologic analysis.

The implanted cells migrated rapidly, throughout the extent of the demyelinated lesions. Within 1 week of implantation, the cells readily traversed the midline to infiltrate the farthest reaches of the demyelinated lesion beds, which often extended over 6 mm in breadth. The migration rate of the cells was hence roughly 1 mm/day, or almost 50 μm/hr within the lesion (Fig. 2B–D).

The surprisingly rapid and extensive migration of the implanted cells raised the possibility that the initial pressure of the injection was contributing to their local dispersal. To ensure that implanted donor progenitor cells were not infiltrating their target lesions as a function of hydraulic pressure, we slowly infused them in 2 μl over 3 min. As an

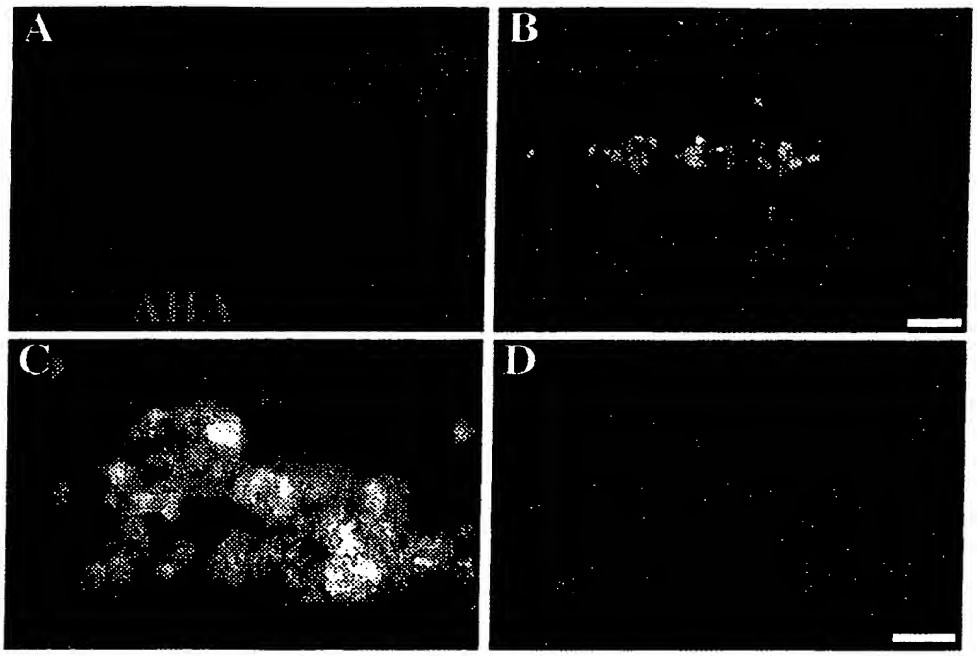

Fig. 5. White matter progenitors express MBP after engraftment to demyelinated foci. Sorted oligodendrocyte progenitor cells derived from the adult human white matter were injected into adult lysolecithin-lesioned rat corpus callosum and were noted to express MBP within 3 weeks thereafter. **A:** In a control animal that received a saline injection, MBP (red) remains absent from the demyelinated central core of the callosum (black) 3 weeks after lesion. **B:** In a rat that received 100,000 sorted progenitor cells unilaterally, human nuclei (green) are surrounded by MBP (red) in the center of the lesion 21 days after implantation (24 days after lysolecithin injection). **C,D:** High-power image showing a cluster of AHA^+ human cells (green) associated with a plethora of MBP^+, myelinating, oligodendrocytic membranes (red). D focuses on the MBP^+ membranes of this field; the oligodendrocytic lamellopodia (red) appear in various stages of ensheathment. Scale bar = 20 μm in B (for A,B); = 5 μm in D (for C,D).

additional control, two rats were injected with 6 μm fluorescent microbeads (Becton-Dickinson, San Jose, CA; 488 nm excitation). In total 100,000 beads (2 μl/3 min) were delivered into callosal foci of lysolecithin demyelination 3 days after lysolecithin injection. The rats were sacrificed 2 hr after surgery, and their brains were cryosectioned and immunostained for MBP. The microbeads were noted to line the cannula track and otherwise remained within a focal deposit at the injection site (Fig. 2C). These findings strongly suggested that pressure injection per se was not associated with significant mechanical dispersion of injected cells.

Normal Brain Is Nonpermissive for Migration of Adult Oligodendrocyte Progenitor Cells

Despite the rapid migration of the implanted progenitor cells throughout the lesion beds (Fig. 2B,C), the cells were typically restricted to regions of demyelination, rarely extending into normal surrounding myelin. Even the few cells that were typically noted to have infiltrated normal myelin appeared to have migrated therein along the extraluminal surfaces of blood vessels. The latter appeared to be limited to vessels that at some point traversed the lesion bed and thereby presented their adventitial surfaces to the migrating implanted progenitors.

On this basis, we asked whether the restriction of implanted progenitors to the lesion site reflected a relative preference for the implanted progenitors to the demyelinated lesion site or whether it instead reflected an absolute impediment of normal white matter to progenitor migration (Jefferson et al., 1997). To this end, we genetically tagged A2B5-sorted adult human WMPCs with CMV-driven EGFP by infecting them in vitro with a lentiviral GFP vector (see Materials and Methods). The fluorescent human WMPCs were then implanted into the intact subcortical white matter of four adult rats to loci including the callosum, hippocampal commissure, and stria medullaris. The fate of the tagged cells was then assessed by sacrificing three of the animals 1 month after implantation and one rat at 2 months. We found that, when injected into intact white matter, the adult progenitor cells remained localized to the implant sites: Whether assessed 4 or 8 weeks after implantation, the cells migrated no farther than the bounds of the initial injectates (Fig. 3).

Adult White Matter Progenitors Differentiate as MBP^+ Oligodendrocytes Upon Xenografting

The engraftment sites each harbored substantial populations of viable cells, many of which expressed CNP protein, indicating their oligodendrocytic maturation.

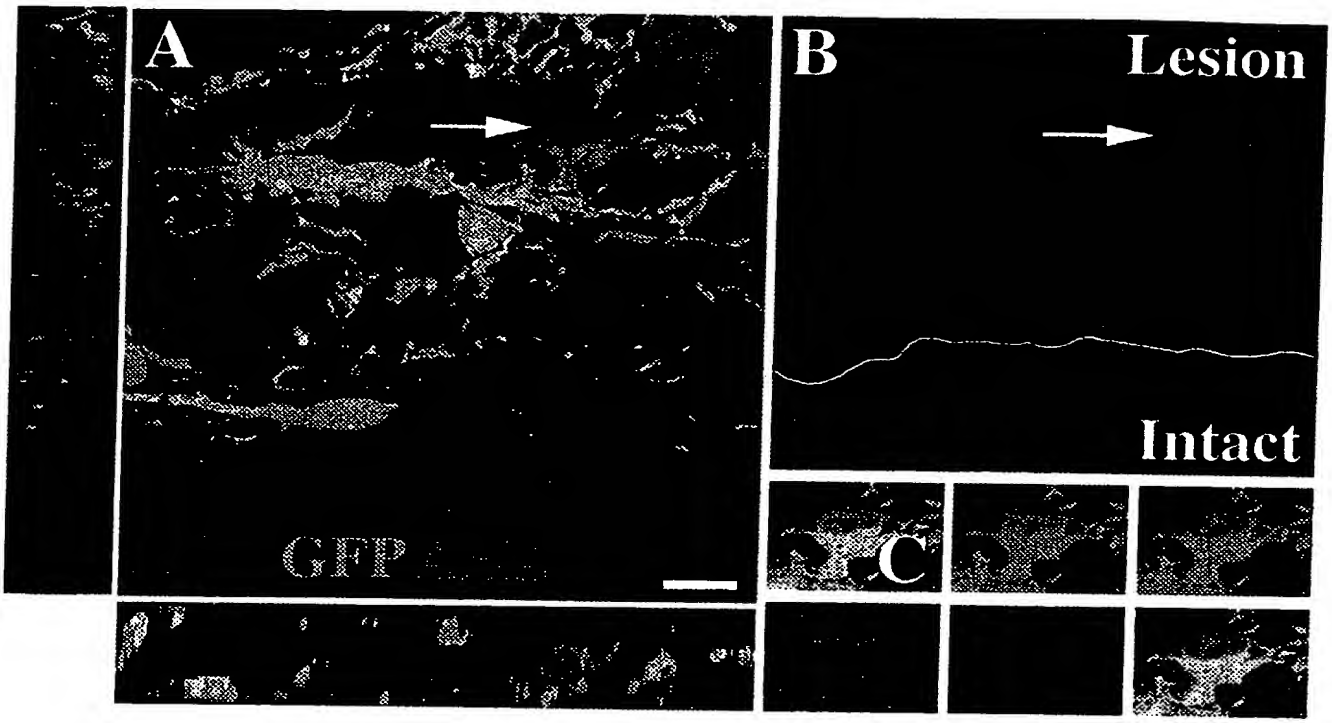

Fig. 6. Genetically tagged adult human-derived white matter progenitors can mature and survive after xenografting. Sorted white matter progenitor cells were tagged with lentivirally delivered GFP, then injected into the lysolecithin-lesioned rat corpus callosum. The recipient animal was killed and its brain immunostained and imaged by confocal microscopy 8 weeks after cell implantation. In **A**, the GFP^+ human progenitors (green) are seen to express human nuclear antigen (AHA; orange), confirming the stability and donor cell restriction of the tag. The side and bottom panels appended to A show orthogonal side views taken through the indicated MBP^+ human donor-derived cell (blue/green; arrow). **B:** Blue color channel of A shows that several of the GFP-tagged human progenitors implanted into the lesion site have differentiated as MBP^+ oligodendroglia (line demarcates lesion border). Many have also matured as astrocytes (data not shown). **C** shows the cell indicated in A, emphasizing its coexpression of lentiviral GFP, human nuclear antigen (AHA), and MBP. Scale bar = 20 μm.

CNP expression typically appeared in implanted adult A2B5-sorted progenitors within 2 weeks of implantation (Fig. 4). By 3 weeks, many had developed expression of MBP (Fig. 5). These human donor-derived cells were noted to project MBP^+ lamellopodia; at low magnification, they were associated with a fine, filamentous array of myelinating fibers. These observations suggested the initiation of progenitor-associated myelinogenesis within the lesion site (Fig. 5). With cyclosporin immunosuppression, we found that these cells could survive for at least 2 months in lysolecithin-demyelinated rat recipients (Figs. 6, 7).

To visualize better the expression of myelin-associated antigens by implanted human progenitors, we also implanted four lysolecithin-lesion animals with lentiviral GFP-tagged human WMPCs. These animals were sacrificed after 4 or 8 weeks, and the fate of the tagged progenitors was assessed histologically. At both time points, GFP-tagged cells were found to have differentiated as admixed populations of oligodendrocytes and astrocytes. No neurons were noted to have arisen from these engrafted progenitors in a matched set of βIII-tubulin-immunostained sections (not shown). Within the lesions, many MBP^+ oligodendrocytes were noted to be GFP tagged and, hence, derived from donor human progenitor cells (Fig. 6). At the lesion borders, a preponderance of $GFAP^+$ GFP-tagged cells was typically noted, indicating the astrocytic differentiation of many of the implanted progenitors (Fig. 7). Few cells of either type migrated beyond the lesion borders.

DISCUSSION

Our previous studies revealed the existence of a distinct population of mitotic oligodendrocyte progenitor cells in the adult human subcortical white matter. These cells are present in both sexes and into senescence and are both ubiquitous and relatively abundant in the adult forebrain white matter. In this study, we found that these oligodendrocyte progenitor cells of the adult human subcortical white matter may also be identified and isolated on the basis of their surface expression of the epitope recognized by MAb A2B5 and that this antigenic phenotype includes those cells defined by CNP2-driven GFP. IMS based on A2B5 expression has allowed us to extract these

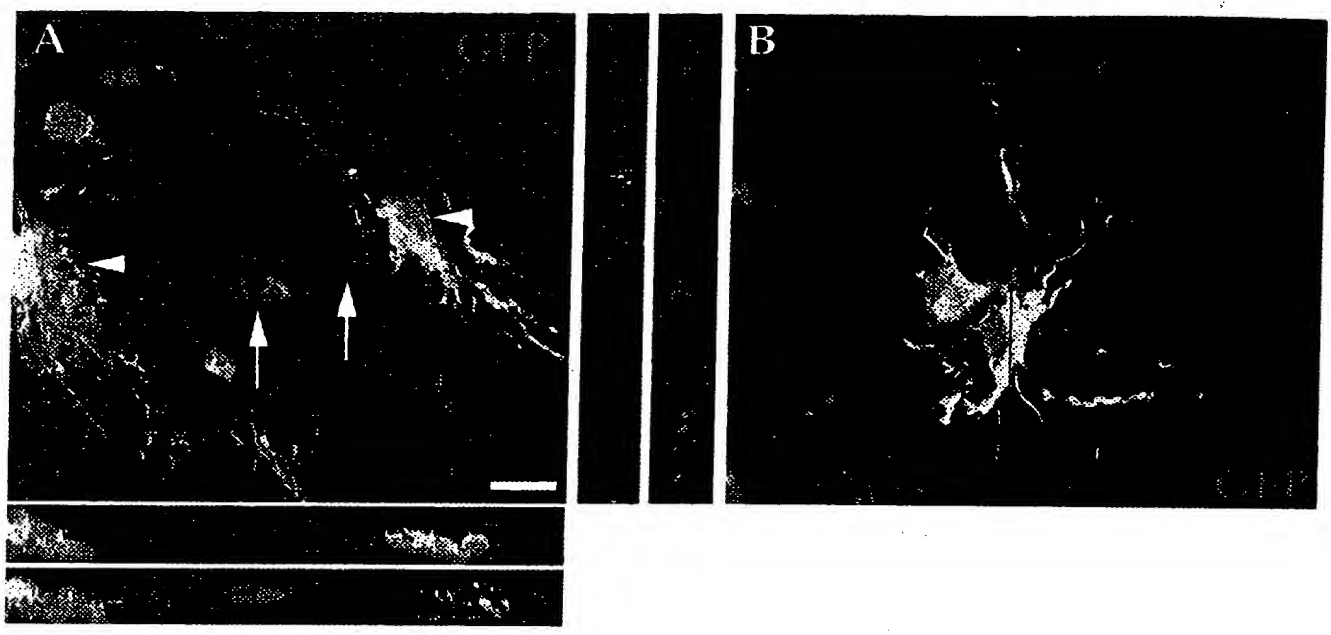

Fig. 7. Both astrocytes and oligodendrocytes arose from implanted adult human white matter progenitors. In **A**, a confocal composite shows two GFP-tagged human (green) MBP^+ (red) oligodendrocytes in the lesion bed of a lysolecithin-injected rat callosum 8 weeks after cell implantation. In addition to the MBP^+ cells (arrows), other human progenitor-derived cells were also present that did not express MBP and that instead manifested astrocytic morphologies (arrowheads). In **B**, immunolabeling for human GFAP (red) revealed that many of the GFP-tagged human progenitors had in fact given rise to astrocytes. Scale bar = 20 μm.

cells from human white matter dissociates in numbers sufficient for experimental xenografting.

The implanted white matter progenitors were found to be highly motile and migratory, infiltrating the demyelinated regions of the white matter over distances up to 1 cm in the week after implantation. However, they were found to avoid normal myelin, which effectively excluded their infiltration. The failure of WMPCs implanted into the normal brain to migrate beyond the injection bed stood in sharp contrast to the fate of otherwise identical cells implanted into lesioned white matter, which migrated rapidly and efficiently throughout the available demyelinated lesion bed. These observations suggest that normal adult white matter is nonpermissive for the migration of adult-derived WMPCs. This restriction appears to be stringent and does not merely reflect demyelinated tissue acting as a preferential substrate for progenitor migration. In a general sense, progenitor cells may be subject to the same types of negative influences on their migration as are axons, whose extension is suppressed in the environment of normal white matter (GrandPre et al., 2000; Chen et al., 2000). However, whereas several myelin-associated moieties that suppress axonal extension, and the axonal receptors for these repulsive ligands, have been identified (Fournier et al., 2001), the operative white matter signals that restrict progenitor cell migration have yet to be determined. The characterization of these repulsive ligands and of their anticipated progenitor cell receptors will likely constitute an important avenue for future study.

The engrafted adult-derived progenitors differentiated largely as oligodendrocytes, and also as astrocytes, and exhibited myelin protein expression in regions of experimental demyelination. The time course of this process was relatively rapid; oligodendrocytic differentiation, as reflected by CNP protein expression, ensued within 2 weeks of donor cell isolation and implantation. Myelinogenesis appeared to follow closely, such that MBP expression attributable to donor cells was evident within 3 weeks of implantation. The efficiency of myelination was difficult to assess in this study, insofar as we did not systematically assess the persistence of axons in these lesions. This caveat notwithstanding, our observations suggest that the introduction of highly enriched preparations of progenitor cells derived from the adult human white matter may permit the structural repair of demyelinated lesions in the adult CNS.

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Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain

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The subcortical white matter of the adult human brain harbors a pool of glial progenitor cells. These cells can be isolated by fluorescence-activated cell sorting (FACS) after either transfection with green fluorescent protein (GFP) under the control of the *CNP2* promoter, or A2B5-targeted immunotagging. Although these cells give rise largely to oligodendrocytes, in low-density culture we observed that some also generated neurons. We thus asked whether these nominally glial progenitors might include multipotential progenitor cells capable of neurogenesis. We found that adult human white-matter progenitor cells (WMPCs) could be passaged as neurospheres *in vitro* and that these cells generated functionally competent neurons and glia both *in vitro* and after xenograft to the fetal rat brain. WMPCs were able to produce neurons after their initial isolation and did not require *in vitro* expansion or reprogramming to do so. These experiments indicate that an abundant pool of mitotically competent neurogenic progenitor cells resides in the adult human white matter.

The adult human subcortical white matter harbors a population of mitotically competent glial progenitors that comprise as many as 3% of its cells[1,2]. These cells may be extracted from brain tissue using FACS after transfection with GFP-encoding plasmids driven by the promoter for *CNP*, an early oligodendrocytic transcript[2,3]. The cells express the immature neural ganglioside recognized by monoclonal antibody A2B5 but do not express more mature markers of glial lineage. We previously noted that when grown at high density, pCNP2:hGFP$^+$ progenitors gave rise to glia, largely oligodendrocytes. Nonetheless, in low-density culture after high-purity FACS, pCNP2:hGFP$^+$ cells often generated βIII-tubulin$^+$ neurons[2]. Because neurogenesis was never observed from pCNP2:hGFP$^+$ cells in higher-density or unsorted cultures, we postulated that the restriction of these progenitor cells to the oligodendroglial phenotype might be an effect of environmental cues rather than a function of autonomous commitment. Once isolated into high-purity, low-density culture, and therefore removed from any paracrine or autocrine influences, human subcortical pCNP2:hGFP$^+$ cells were able to generate neurons as well as glia[2]. It was subsequently reported[4] that glial progenitors from the postnatal rat optic nerve could also generate neurons after serum- or bone morphogenetic protein–induced phenotypic instruction and basic fibroblast growth factor (bFGF)–stimulated expansion. Similar work showed that progenitor cells of the adult rat forebrain parenchyma could also generate neurons after prolonged *in vitro* expansion in bFGF[5]. Taken together, these findings indicated that glial progenitor cells might retain substantial phenotypic plasticity.

We asked whether some fraction of the nominally glial progenitors of the adult human subcortical white matter might actually be parenchymal neural stem cells. Specifically, we asked whether single, sorted WMPCs could generate multiple neural phenotypes, and if so, whether they were capable of expansion and self-renewal. In addition, we investigated whether this process requires de-differentiative reprogramming to an intermediate phenotype, or whether simply removing these cells from their local environment and mitotically expanding them in bFGF might suffice to permit these cells to act as multipotential progenitors. In doing so, we tested the hypothesis that the phenotypic plasticity of adult WMPCs might be tonically restricted by the adult parenchymal environment, rather than irreversibly lost with development.

WMPCs were isolated by *CNP*- and A2B5-based sorting

White matter was dissected from surgical samples taken at the time of temporal lobectomy for epilepsy, aneurysm, and post-traumatic decompression ($n = 21$). The tissues were dissected free of adjacent cortex and ventricular epithelium, and enzymatically dissociated to single-cell suspension as described[2]. The dissociates were plated onto laminin (100 μg/ml) in DMEM/F12/N1 supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and platelet-derived growth factor (PDGF)-AA (20 ng/ml). To identify oligodendrocyte progenitors, the dissociates were trans-

fected with pCNP2:hGFP, the transcription of which results in GFP expression by oligodendrocyte progenitor cells[2].

To avoid both the temporal lag between transfection and GFP expression and the inefficiency of plasmid transfection, cultures were also sorted on the basis of A2B5 surface immunoreactivity, which can serve as a surrogate marker for pCNP2:hGFP$^+$ WMPCs *in vitro*[2]. Immunostaining showed that 84 ± 8.3% of pCNP2:GFP$^+$ cells expressed A2B5 (ref. 2). GFP-based FACS gated 0.49 ± 0.15% of all white-matter cells as pCNP2:hGFP$^+$ (mean ± s.e.m.; $n = 3$ patients; Fig. 1*a*). Matched cultures transfected with pCMV:GFP had a net transfection efficiency of 13.1%. Thus, the predicted incidence of pCNP2:hGFP$^+$ cells in the white matter was 3.7% (= 1 + 0.131 × 0.49), consistent with our prior estimates of the incidence of this phenotype[2]. From the same samples, A2B5-based FACS gated an average of 3.1 ± 0.7% ($n = 3$) of the white-matter cell population (Fig. 1*b*). The greater than six-fold increase in net yield when A2B5 was used (3.1% versus 0.49%) reflected the higher efficiency of A2B5 immunodetection relative to pCNP2:hGFP plasmid transfection. On this basis, we used immunomagnetic sorting (IMS) to select A2B5$^+$ cells from adult white-matter dissociates. By IMS, the incidence of A2B5-sorted cells in white matter dissociates was 3.6 ± 0.3% ($n = 21$) with a median of 3.1%. This improved yield was accomplished with no appreciable loss of cell-type specificity, in that the A2B5$^+$ cells overlapped entirely with the sort profiles of pCNP2:hGFP$^+$ cells and each isolate generated O4$^+$ oligodendrocytes with similar efficiency (Fig. 1*c–f*). Thus, A2B5-based FACS and IMS identified WMPCs homologous to those recognized by pCNP2:GFP-based FACS, while permitting higher-yield isolation of these cells.

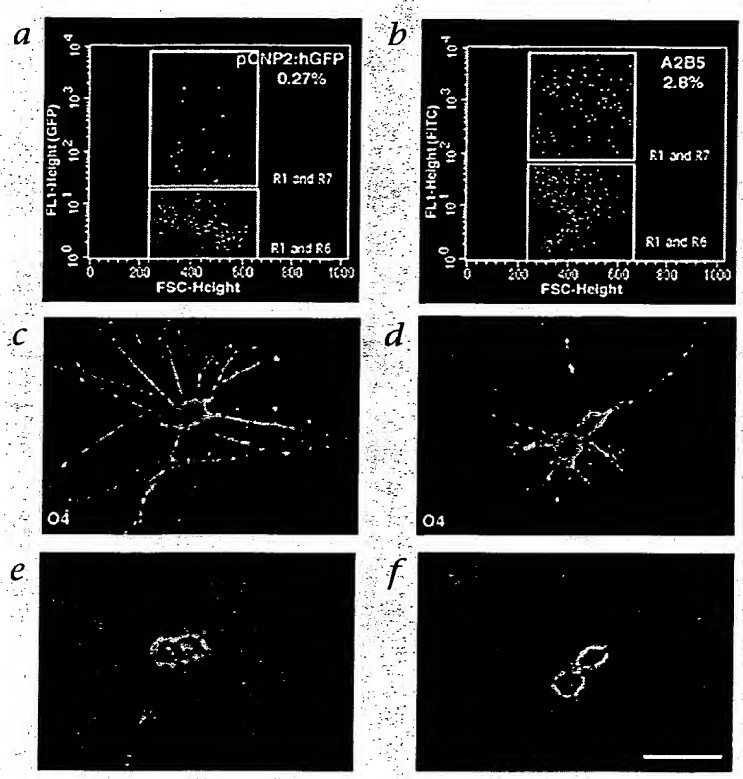

Fig. 1 A2B5-based FACS selects oligodendrocyte progenitor cells. ***a*** and ***b***, FACS graphs showing the extraction of pCNP2:hGFP$^+$ (*a*) and A2B5$^+$ (*b*) WMPCs from an adult human white-matter dissociate. Forward scatter (FCS), a measure of cell size, is plotted against fluorescence intensity (Fl-1). When pCNP2:hGFP- and A2B5-based sorts were directly compared, their plots showed overlapping profiles, but A2B5$^+$ cells were >6-fold more abundant than their pCNP2:hGFP$^+$ counterparts, reflecting the higher efficiency of A2B5 surface tagging. ***c–f***, Progenitors sorted by pCNP2:hGFP (*c* and *e*) and A2B5 (*d* and *f*) gave rise to O4$^+$ oligodendrocytes. A2B5-based surface antigen sorting may thus be used as a higher-yield alternative to pCNP2:hGFP transfection–based FACS for isolating WMPCs. Scale bar, 24 μm.

Adult WMPCs gave rise to multipotent neurospheres

To assess the expansion capacity of pCNP2:hGFP- and A2B5-sorted cells, we propagated sorted isolates of each in suspension[6–8]. The cells were distributed into 24-well plates at 50,000 cells per 0.5 ml in serum-free media (SFM) supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and PDGF-AA (20 ng/ml), a combination that permits the expansion of human WMPCs[2]. Seven days later, the cells were switched to SFM with bFGF alone (20 ng/ml)[8]. Over the next 10 d, neurospheres—spherical masses of cells that expand from single parental progenitors—arose in these cultures, such that by 3 weeks after sorting, there were 84.8 ± 9.0 spheres/well ($n = 4$ patients). These neurospheres were typically >150 μm in diameter and included 46.5 ± 8.2 cells/sphere (Fig. 2*a* and *b*). Thus, single WMPCs of the adult human brain were capable of generating neurospheres.

To establish the lineage potential of single adult human WMPCs, we dissociated the resultant primary neurospheres and passaged them into new wells. Alternatively, some were plated onto substrate to permit their differentiation. Immunostaining showed that both pCNP2:hGFP$^+$ and A2B5$^+$ progenitor-derived spheres gave rise to all major neural phenotypes (Fig. 2*d* and *e*). Among those cells passaged from primary spheres, secondary spheres were observed to arise within two weeks after passage. After expansion, these secondary spheres were similarly plated on substrate, raised for one to two weeks and fixed. Immunolabeling confirmed that virtually all secondary spheres generated both neurons and glia together (Fig. 2*c* and *e*). In addition, when the mitotic marker BrdU was added to A2B5-sorted cells, BrdU-incorporating neurons, oligodendrocytes and astrocytes all emerged from the spheres generated (Fig. 2*f–i*). The persistence of mitotic neurogenesis and gliogenesis by single spheres indicated that they contained cycling multipotential cells. The secondary spheres were probably of clonal origin, given the low plating density of the single cells from which each was derived and the fact that the sphere-forming cells originated from primary spheres that had themselves expanded from single-cell dissociates. These data indicate that single progenitor cells of the adult human white matter are both clonogenic and multipotent.

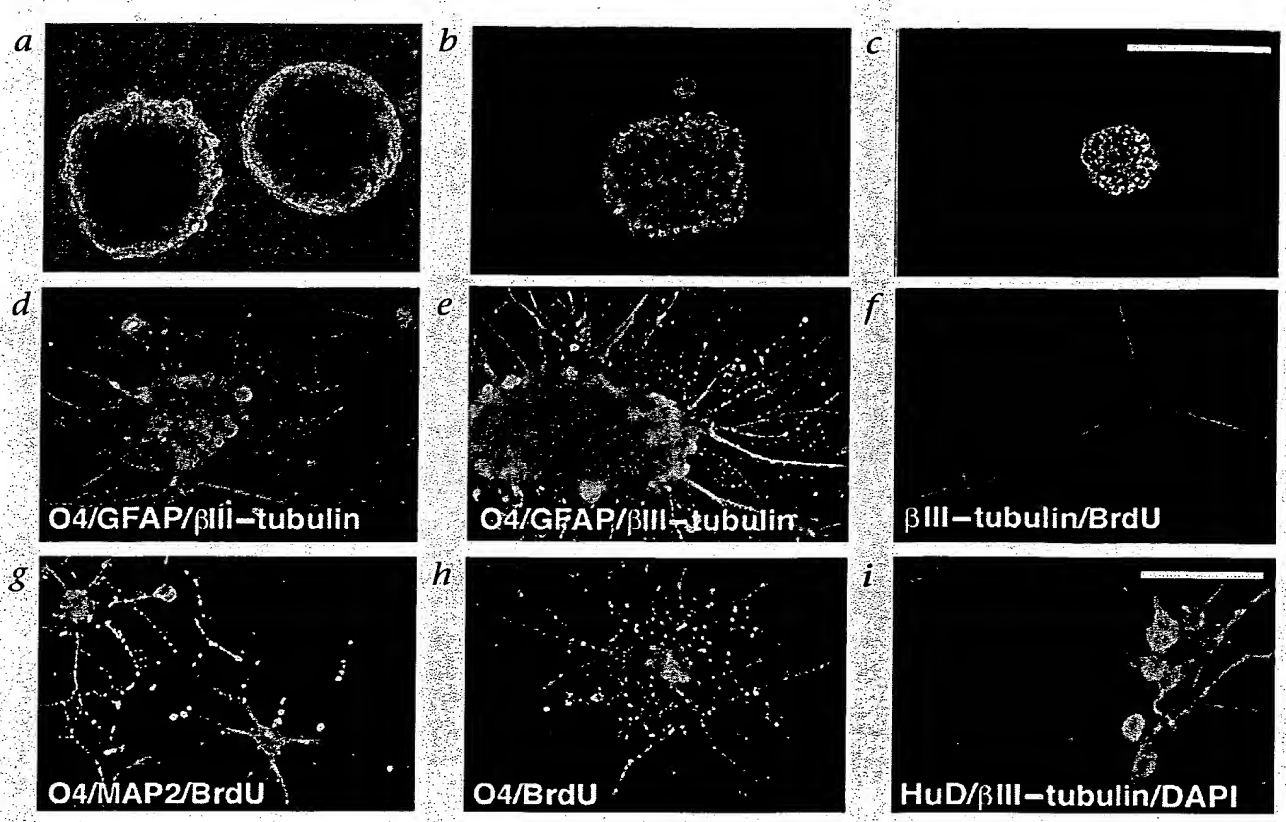

Fig. 2 Adult human WMPCs give rise to multipotential neurospheres. ***a***, First-passage spheres generated from A2B5-sorted cells 2 weeks after sorting. ***b***, First-passage spheres arising from pCNP2:hGFP-sorted cells, at 2 weeks. ***c***, Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. ***d***, Once plated onto substrate, the primary spheres differentiated into βIII-tubulin$^+$ neurons (red), GFAP$^+$ astrocytes (blue) and O4$^+$ oligodendrocytes (green). ***e***, Neurons (red), astrocytes (blue) and oligodendrocytes (green) arose similarly from spheres derived from pCNP2:GFP-sorted WMPCs. ***f–h***, BrdU incorporation (blue) showed that new neurons (*f*, βIII-tubulin (red); *g*, MAP2 (red)) and oligodendrocytes (*h*, O4 (green)) were generated *in vitro*. ***i***, βIII-tubulin$^+$ neurons (green) co-expressed neuronal Hu protein[40,41] (red), yielding double labeling (yellow). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm (*a–e*) or 40 μm (*f–i*).

Single WMPCs remained multipotential with passage

The serial propagability of sorted WMPCs from neurospheres in low-density dissociates suggested the clonal derivation of each individual sphere[9–11]. To further validate the clonal origin of neurons and glia arising within single spheres, we used lentiviral GFP to genetically tag and follow single WMPCs. A2B5$^+$ cells were tagged, 2–5 d after sorting, with a lentivirus expressing GFP under cytomegalovirus (CMV) promoter control[12–14]. At 10 PFU/cell, 23% of the cells expressed GFP by one week after sorting, yielding a mixture of GFP$^-$, GFP$^+$ and mixed spheres in the resultant cultures (Fig. 3*a–b*). These primary spheres were triturated two to four weeks later to single-cell suspensions and passaged into bFGF at ~3,000 cells/well. Under these conditions, 40.8 ± 12.9 secondary spheres/well were generated, indicating a clonogenic cell incidence of 1.3% (n = 5). Of these secondary spheres, 47.2 ± 10.8% contained only GFP$^+$ cells (Fig. 3*c–d*) whereas 30.9 ± 6.9% harbored no GFP$^+$ cells. The relative uniformity of GFP expression, or lack thereof, among the cells within a given sphere indicated that most spheres were clonally derived ($P < 0.005$ by χ^2 analysis). This tested the null hypothesis that the spheres arose from non-clonal aggregation of two or more cells, each of which was equally likely to be GFP$^+$ or GFP$^-$. When the single spheres were plated onto polyornithine and fibronectin and their outgrowth assessed two weeks later, all gave rise to both neurons and glia (Fig. 3*e–g*). Because most secondary spheres were likely to have been clonally derived, and all included neurons as well as glia (38 of 38 spheres; n = 4 samples), single WMPCs must have given rise to neurons and glia together.

We next asked if the neurogenic capacity and multilineage competence of WMPCs were maintained with passage. Primary spheres were raised serially in bFGF/NT3/PDGF-AA for 7 d, DMEM/F12/N1 with 15% serum/PDGF-AA for 4 d, and serum-free DMEM/F12/N1 with bFGF for 10 d. Cells were then dissociated and replated in bFGF at 3,000 cells/well in a 24-well plate. Secondary spheres arose within two weeks from 1.1 ± 0.3% of these cells (n = 8). After more than two weeks of further expansion, the secondary spheres were plated on polyornithine and fibronectin and were fixed and immunostained two weeks later (seven to nine weeks after sorting). Whereas primary spheres consisted of 21.7 ± 4.3% βIII-tubulin$^+$ neurons, 17.7 ± 3.9% glial fibrillary acidic protein (GFAP)$^+$ astrocytes and 46.7 ± 5.9% O4$^+$ oligodendrocytes (n = 3), secondary spheres consisted of 16.0 ± 2.5% neurons, 19.3 ± 3.2% astro-

cytes and 46.4 ± 2.4% oligodendrocytes (n = 3). Most of the neurons were GABAergic, by virtue of their expression of glutamic acid decarboxylase-67 (GAD67) (Fig. 4*a–c*). Because the relative proportions of neurons, oligodendrocytes and astrocytes in secondary spheres were similar to those in primary spheres, we concluded that WMPCs retained multilineage competence with expansion.

WMPC-derived neurons become functionally mature

The calcium responses and membrane currents of WMPC-derived neurons were assessed to establish their ability to respond to depolarizing stimuli. Primary spheres (n = 12 fields, derived from 3 brains) were plated on fibronectin to permit neuronal outgrowth, and assessed 14 d later for their calcium responses to depolarizing stimuli. The cultures were then loaded with the calcium indicator dye Fluo-3 and serially exposed to both 100 μM glutamate and 60 mM potassium during confocal microscopy. Astrocytic responses to depolarization were minimal under these culture conditions, as previously noted. In contrast, neuron-like cells displayed rapid, reversible, >100% elevations in cytosolic calcium in response to potassium, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 4*d–f*). The neuronal phenotype of these cells was then validated by immunostaining for βIII-tubulin.

We then asked whether WMPC-derived neurons would be able to develop the fast sodium currents and action potentials characteristic of electrophysiologically competent neurons. We used whole-cell patch-clamp recording during current stimulation to assess the response of WMPC-derived neurons that arose from plated secondary spheres derived from A2B5-sorted isolates. A total of 58 WMPC-derived fiber-bearing cells were recorded, in 5 cultures derived from 3 patients. Of these, 13 showed voltage-activated sodium ion currents (I_{Na}) of >100 nA, and 7 had I_{Na} > 600, compatible with the fast sodium currents of neuronal depolarization[15,16]. Accordingly, whereas two of five cells with I_{Na} > 800 generated stimulus-evoked action potentials (Fig. 4*g–h*), none did so with I_{Na} < 800. In addition, none of 26 morphologically non-neuronal cells showed substantial (≥100 pA) current-induced sodium currents. Together, these results indicated that neurons arising from adult human WMPCs developed mature electrophysiologic functions, including both fast sodium currents and action potentials.

WMPCs generated neurons without reprogramming

Glial progenitor cells from the postnatal rat optic nerve can generate neurons, under conditions that have been described as 'reprogramming' glial progenitors to multilineage competence[4]. In that study, neurogenesis was achieved by first instructing the cells to an intermediary astrocytic lineage using either serum or bone morphogenetic protein-2, followed by bFGF-stimulated mitogenesis. We asked whether such reprogramming steps are required for the generation of neurons from adult human WMPCs, or whether simple expansion under minimal conditions *in vitro*, with the removal of these cells from their environment, might be sufficient to permit neurogenesis by these cells. Sorted A2B5$^+$ cells were cultured in several permutations of mitogenic and differentiative conditions to identify the minimal conditions permissive for lineage diversification. We compared the phenotypes generated under three conditions: (i) bFGF/NT3/PDGF-AA in SFM (composed of DMEM/F12/N1) for 7 d, followed by 15% FBS/ PDGF-

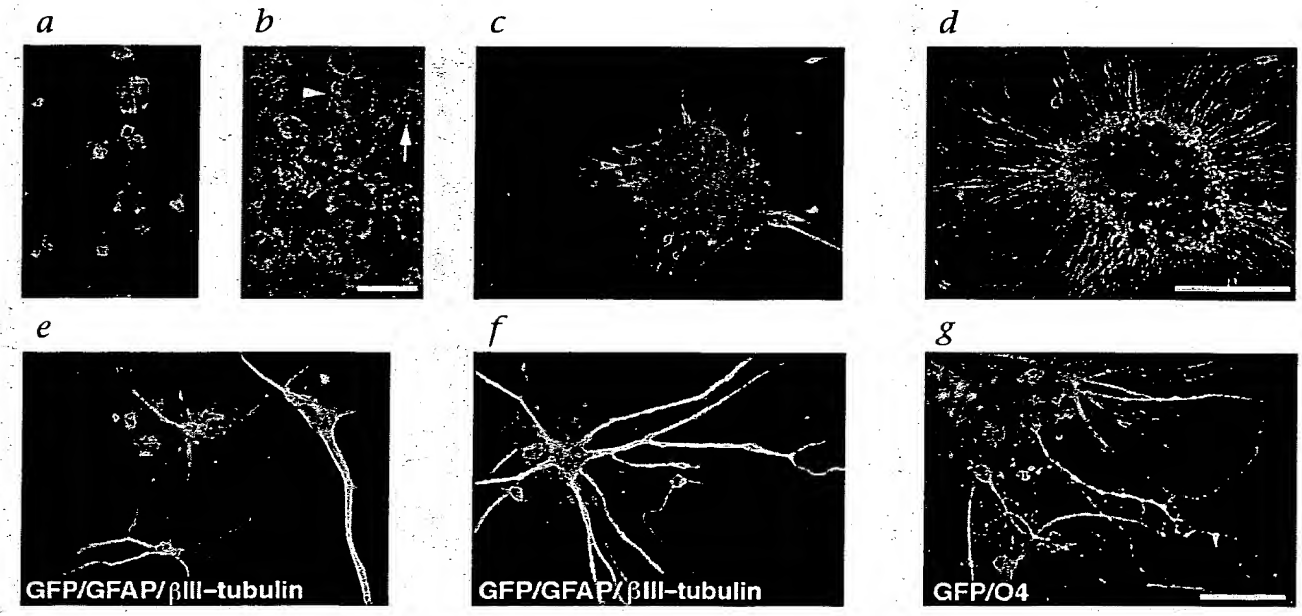

Fig. 3 Single lentiviral GFP-tagged WMPCs generated neurons and glia. A2B5-sorted WMPCs were infected with a lentivirus encoding enhanced GFP[14], 5 d after sorting. ***a*** and ***b***, Secondary spheres subsequently derived from infected cells harbored either GFP-tagged cells (arrowhead), untagged cells (arrow) or, less commonly, both. ***c*** and ***d***, GFP$^+$ secondary sphere 1 week after plating. ***e*** and ***f***, βIII-tubulin$^+$ neurons (red) and GFAP$^+$ astrocytes (blue) arising from a single clonally derived GFP$^+$ secondary sphere. ***g***, GFP$^+$ (green) and O4$^+$ (red) oligodendrocytes arising from a secondary sphere. Scale bars, 100 μm (*a* and *b*), 60 μm (*c* and *d*) or 40 μm (*e–g*).

AA for 4 d and SFM with bFGF for two weeks; (ii) bFGF/NT3/PDGF-AA in SFM continuously for three weeks; and (iii) bFGF alone in SFM for three weeks. The first condition was intended to promote initial differentiation in serum, whereas the latter two groups were designed to skip this glial differentiative step[4].

The A2B5-sorted progenitors yielded spheres under each of these conditions; however, both the number of spheres and the percentage of neurons generated by each differed as a function of treatment. Cultures maintained in base media alone or in bFGF-supplemented media had 5.9 ± 1.7% and 7.2 ± 2.1% βIII-tubulin$^+$ neurons, respectively (n = 3 patients). When matched WMPC-derived spheres were sequentially raised in bFGF/NT3/PDGF-AA with 15% serum and bFGF, 18.2 ± 2.2% of the cells were βIII-tubulin$^+$ (Fig. 5*a*). A similar proportion of neurons (22.5 ± 1.9%; n = 3) was generated by those neurospheres maintained in SFM with bFGF/NT3/PDGF-AA. Serum exposure was therefore not required for A2B5$^+$ cells to generate neurons. Indeed, no specific signals seemed necessary for neuronal instruction, besides those provided by PDGF and NT3. These data indicated that antecedent astrocytic differentiation was not a necessary prerequisite to neurogenesis by adult WMPCs. These cells required neither prolonged mitogenic expansion, nor specific dedifferentiation steps, to generate neurons as well as glia[4].

Although both PDGF and NT3 promote oligodendrocyte production by glial progenitors of the rat optic nerve[17,18], each can induce neuronal differentiation in less-committed hippocampal and ventricular zone neural progenitors[19,20]. As such, their neurogenic effects on adult WMPCs may reflect the relatively undifferentiated state of these cells.

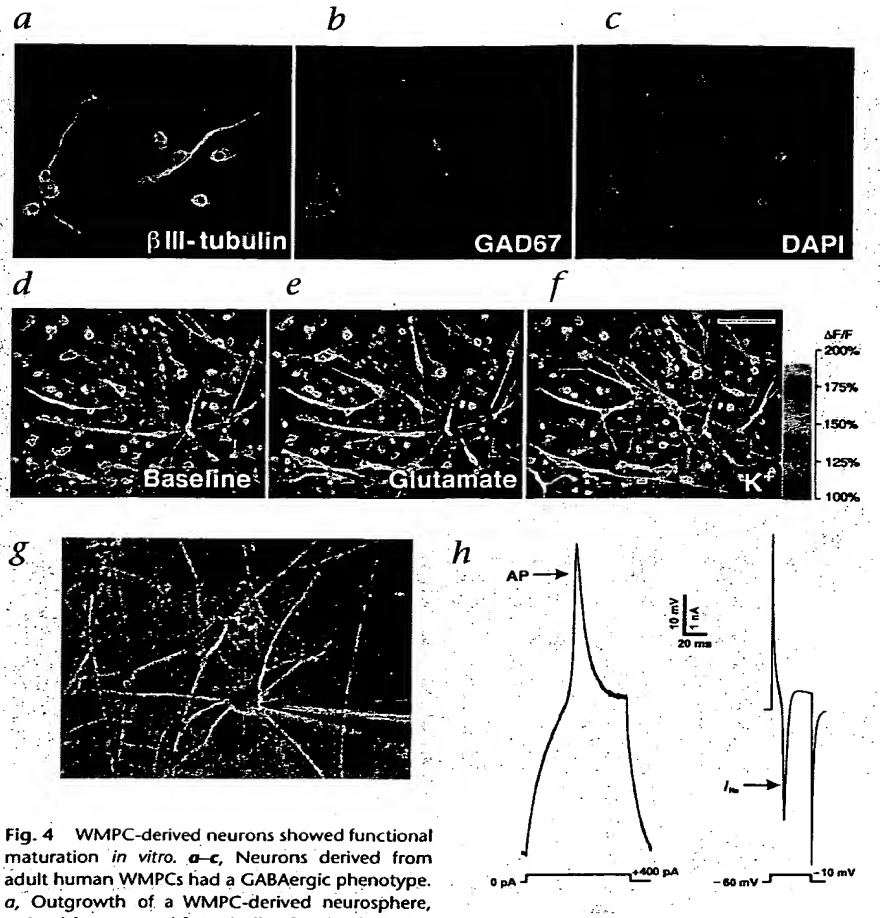

Fig. 4 WMPC-derived neurons showed functional maturation *in vitro*. ***a–c***, Neurons derived from adult human WMPCs had a GABAergic phenotype. *a*, Outgrowth of a WMPC-derived neurosphere, stained for neuronal βIII-tubulin after 35 d *in vitro*. *b*, Immunostaining showed that all 9 neurons in the field were GAD67$^+$ and were thus likely to be GABAergic. *c*, DAPI nuclear counterstaining showed the abundance of cells in the field. ***d–f***, WMPC-derived neurons developed neuronal Ca^{2+} responses to depolarization. *d*, WMPC-derived cells loaded with the calcium indicator dye Fluo-3, 10 d after plating of first-passage spheres derived from A2B5-sorted white matter (35 d *in vitro* total). Many fiber-bearing cells of both neuronal and glial morphologies are apparent. *e*, The same field after exposure to 100 μM glutamate. *f*, The same field after exposure to a depolarizing stimulus of 60 mM KCl. Rapid, reversible, >100% elevations in cytosolic calcium occurred in response to K^+, consistent with the activity of neuronal voltage-gated calcium channels. Scale bar, 80 μm. ***g*** and ***h***, Whole-cell patch-clamp experiments detected voltage-gated sodium currents and action potentials in WMPC-derived neurons. *g*, Representative cell, 14 d after plating of first-passage sphere derived from A2B5-sorted white matter. The cell was patch clamped in a voltage-clamped configuration and its responses to current injection were recorded. *h*, Action potentials (AP) were noted after positive current injection, at I_{Na} >800 pA (left tracing). The fast negative deflections noted after depolarization steps are typical of the voltage-gated sodium currents of mature neurons (right).

Only a fraction of A2B5$^+$ cells were clonogenic

We next assessed the incidence of clonogenic and multipotential progenitor cells within the larger pool of A2B5-sorted white-matter cells. We first assessed whether either the survival or the mitotic competence of adult human WMPCs were dependent on density, by assessing the limiting dilution at which clonogenic progenitors could be obtained from A2B5-sorted white-matter dissociates. A2B5$^+$ cells were plated immediately after sorting, at densities ranging from 100,000 to 1,000 cells/ml (0.5 ml cell suspension per well of a 24-well plate), in basal media supplemented with bFGF/NT-3/PDGF-AA. Under these conditions, the incidence of clonogenic progenitors was a curvilinear function of the sorted cell density (R^2 = 0.978; Fig. 5*b*). Whereas 186 ± 7.6 spheres were generated at a density of 100,000 cells/ml (0.4%; n = 5 patients), only 6.5 ± 2.7 were noted at 10,000 cells/ml (0.1%) and no sphere generation was noted at or below 5,000 cells/ml. Thus, the expansion of purified WMPCs was density dependent and optimal at 50,000–100,000 cells/ml. Densities higher than the optimal range seemed to promote terminal differentiation of the progenitors.

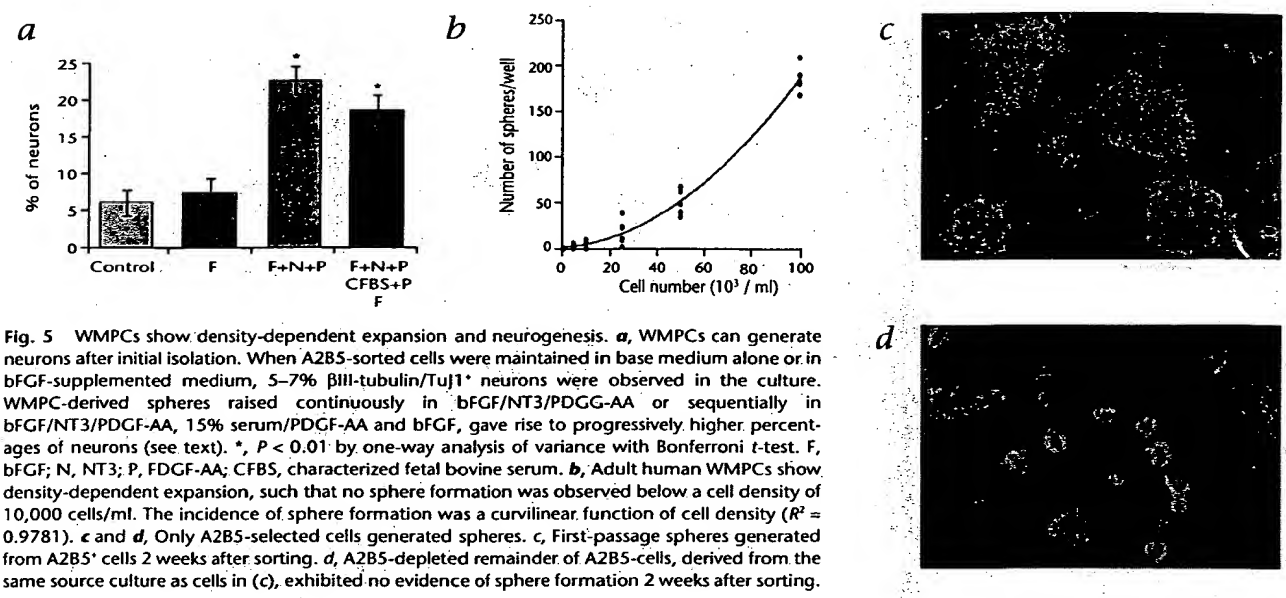

Fig. 5 WMPCs show density-dependent expansion and neurogenesis. *a*, WMPCs can generate neurons after initial isolation. When A2B5-sorted cells were maintained in base medium alone or in bFGF-supplemented medium, 5–7% βIII-tubulin/TuJ1⁺ neurons were observed in the culture. WMPC-derived spheres raised continuously in bFGF/NT3/PDGG-AA or sequentially in bFGF/NT3/PDGF-AA, 15% serum/PDGF-AA and bFGF, gave rise to progressively higher percentages of neurons (see text). *, $P < 0.01$ by one-way analysis of variance with Bonferroni *t*-test. F, bFGF; N, NT3; P, FDGF-AA; CFBS, characterized fetal bovine serum. *b*, Adult human WMPCs show density-dependent expansion, such that no sphere formation was observed below a cell density of 10,000 cells/ml. The incidence of sphere formation was a curvilinear function of cell density (R^2 = 0.9781). *c* and *d*, Only A2B5-selected cells generated spheres. *c*, First-passage spheres generated from A2B5⁺ cells 2 weeks after sorting. *d*, A2B5-depleted remainder of A2B5-cells, derived from the same source culture as cells in (*c*), exhibited no evidence of sphere formation 2 weeks after sorting.

To assess whether clonogenic WMPCs were restricted to the A2B5⁺ population, we also cultured the A2B5-depleted pool remaining after each sort. A2B5-depleted cultures did not give rise to any passageable neurospheres at any of the cell densities assessed over the range of 1,000–100,000 cells/ml (Fig. 5*d*). On the basis of these studies, we concluded that only a fraction of white-matter A2B5⁺ cells are actually clonogenic and multipotential progenitors, although all clonogenic WMPCs are A2B5⁺.

Adult WMPCs showed limited self-renewal

We next sought to define the extent to which WMPCs were self-renewing by assessing the extent to which WMPC-derived neurospheres were capable of repetitive passage. Primary spheres were raised from three patients at an optimal initial density of 100,000 cells/ml, under the conditions identified as most supportive of multilineage expansion (bFGF/NT3/PDGF-AA in DMEM/F12/N1). One month later, the spheres were dissociated and replated. Secondary spheres were generated and were replated one month later at 1×10^4–5×10^4 cells/ml. These cultures gave rise to tertiary spheres over the following month, though with less efficiency and a smaller volumetric expansion than secondary spheres. Attempts at propagating these spheres as quaternary spheres, after additional dissociation, were generally unsuccessful. Given an apparent cell doubling time of 3–4 d (data not shown) and monthly passages spanning 8–10 doublings, we estimated that the tertiary spheres assessed one month after the last passage underwent a minimum of 16–24 and no more than 30 doublings. This is well below the number of doublings of which tissue-derived stem cells are typically thought capable.

Our inability to successfully passage these cells beyond 16–24 doublings called into question their ability to self-replicate for extended periods of time *in vitro*. Their limited replicative competence contrasted with that of neural progenitors sorted from the fetal human ventricular zone, which may be readily passaged for >60 doublings under analogous culture conditions[21]. Such self-renewal capacity has been ascribed to sustained telomerase activity in a number of developing systems, including the fetal human forebrain[22,23]. To assess whether the apparently finite proliferative potential of adult human WMPCs reflected a lack of telomerase activity, telomerase levels were assessed using the telomerase reverse transcriptase activity protocol (TRAP) assay[23,24]. We did not detect any telomerase activity in primary or secondary WMPC-derived spheres, despite high-level activity in a variety of positive controls (see Supplementary Fig. 1 online). Their lack of extended replicative potential, coupled with their lack of telomerase activity, suggests that adult WMPCs might constitute a pool of multipotential progenitors with a finite capacity for mitotic expansion, transitional between tissue-restricted stem cells and phenotypically committed progenitors.

WMPCs produced neurons and glia after fetal xenograft

We next assessed whether WMPCs were multipotential *in vivo* as well as *in vitro* by evaluating their fate after engraftment to embryonic stage (E)17 fetal rat brains. Some A2B5-sorted cells were transplanted 24–48 h after sorting to assess their lineage potential upon initial isolation. These cells were maintained only in SFM during the period between isolation and xenograft and were never exposed to any exogenous growth factors. Other cells were transplanted 10 d after sorting, after maintenance in bFGF/NT3/PDGF-AA for 4 d and 15% serum/PDGF-AA followed by bFGF, for 3 days each. All donor cells were administered into E17 rat embryos by intraventricular injection at 10^5 cells/animal. The recipients were killed and fixed four weeks after birth to evaluate the fate of the implanted human cells. Human donor cells were identified by immunolabeling of brain sections for human nuclear antigen (HNA).

In rats implanted with propagated WMPCs (Fig. 6) and their counterparts injected with acutely isolated WMPCs (see Supplementary Fig. 2 online), donor-derived migrants co-expressing HNA with either nestin or doublecortin[25] were found in the host olfactory subpendyma and hippocampus (Fig. 6*a* and

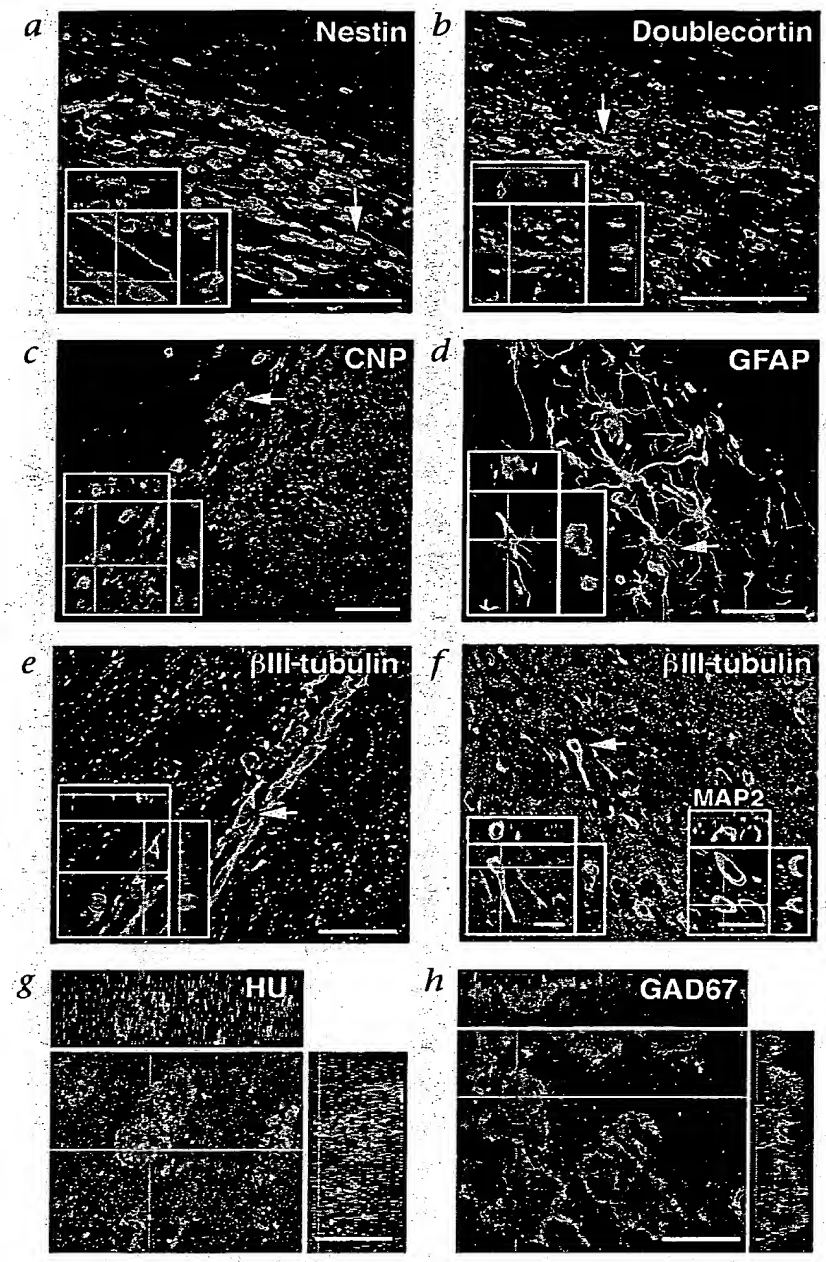

Fig. 6 WMPCs engrafted into fetal rats give rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and killed 1 month after birth. Cells were maintained in culture for 10 d before implanting. *a* and *b*, Nestin$^+$ (*a*) progenitors and doublecortin$^+$ (*b*) migrants (red) each co-expressing HNA (green) in the hippocampal alvius. *c*, CNP$^+$ (red) HNA$^+$ (green) oligodendrocytes, found exclusively in the corpus callosum. *d*, Low-power image of GFAP$^+$ (green) HNA$^+$ (red) astrocytes (yellow, double-positive) along the ventricular wall. *e*, βIII-tubulin$^+$ (green) and HNA$^+$ (red) neurons migrating in a chain in the hippocampal alvius. *f*, βIII-tubulin$^+$ and MAP2$^+$ (inset) neurons in the striatum, adjacent to the rostral migratory stream (green, bIII-tubulin and MAP2; red, HNA; yellow, double-stained human nuclei). *g*, Hu$^+$ (red) HNA$^+$ (green) neuron in the septum. *h*, GAD67$^+$ (red) HNA$^+$ (green) striatal neuron. Insets (*a–f*) show orthogonal projections of a high-power confocal image of each identified cell (arrow). Scale bars, 40 μm (*a–e*) or 20 μm (*f–h*).

b). In addition, abundant populations of HNA$^+$ βIII-tubulin$^+$ neurons were found in the olfactory subependyma and rostral migratory stream as well as in the hippocampal alvius (Fig. 6*e*). WMPC-derived neurons were also observed in the neostriatum, indicating striatal neuronal differentiation on the part of some xenografted WMPCs (Fig. 6*f*). These data showed that engrafted adult human WMPCs could integrate into the forebrain subventricular zone as neuronal progenitor cells that then gave rise to both granule and striatal neurons. Human WMPC-derived GFAP$^+$ astrocytes and CNP$^+$ oligodendrocytes were also common in recipient brains and were found primarily along the ventricles or in the subcortical white matter (Fig. 6*c* and *d*). Thus, adult human WMPCs showed context-dependent differentiation after xenograft to the developing rat brain and were competent to do so upon acute isolation, without the benefit of humoral instruction *in vitro*.

Discussion

These observations suggest that the WMPCs of the adult human forebrain include multipotential progenitor cells, capable of a finite and limited degree of expansion and self-renewal. These cells remain competent to respond to local instructive cues, with a wide range of lineage choices, upon xenograft as well as *in vitro*. They are readily able to give rise to neurons and glia once they are removed from their native white-matter environment. The freshly isolated adult WMPCs in our study did not require prolonged expansion to undergo neurogenesis *in vitro*, and seemed immediately competent to generate neurons upon xenograft to the developing brain.

Previous studies of the adult rat brain have identified parenchymal progenitor cells that are able to give rise to neurons and glia after a number of cell doublings, in the presence of bFGF[8]. In addition, nominally committed glial progenitor cells derived from the neonatal rat optic nerve have also been reported to give rise to neurons and oligodendrocytes[4]. The lineage diversification of these cells seems to require a humorally directed reprogramming of their phenotype, with the induction of an astrocytic intermediary on the way to neurogenesis. In the present study, adult human WMPCs did not seem to require any such reprogramming or transdifferentiation to achieve multilineage competence. Similarly, they did not seem to pass through an intermediate astrocytic stage before generating neurons, oligodendrocytes and astrocytes. Indeed, after their acute isolation and xenograft, A2B5-defined WMPCs were able to

generate all major neural phenotypes *in vivo* and *in vitro*, without any exogenous growth factor exposure. Nevertheless, because an average of 7% of A2B5-sorted white-matter cells co-expressed GFAP (data not shown), it is possible that some WMPCs exhibit astroglial features at some point during their ontogeny, much like subventricular neural progenitor cells[26,27]. This categorization notwithstanding, our results suggest that the WMPCs of the adult human brain are fundamentally tissue-specific progenitor cells that are tonically restricted to glial lineage by the local parenchymal environment, and do not require specific phenotypic reprogramming for neuronal differentiation.

These data suggest that adult human WMPCs constitute a population of parenchymal glial progenitor cells whose *in situ* fate is restricted by the local white-matter environment. Yet the progenitor cell pool of the adult white matter may be heterogeneous, and it is not clear whether all WMPCs have the same ontogeny or fate potential[28–30]. A minority of multipotential progenitor cells might still persist among a larger pool of more fundamentally lineage-restricted glial progenitors[8]. These parenchymal multipotent progenitors may constitute a relatively rare subpopulation, more akin to persistent stem cells than to any lineage-restricted derivatives[31,32]. In this regard, although we did not detect telomerase activity in sorted WMPCs, if the clonogenic portion of these represents only a small fraction of the total progenitor pool, then their numbers might have been below the detection threshold of our TRAP assay. Further study of the heterogeneity of the white-matter progenitor cell population, and of the lineage competence of its constituent phenotypes, will be needed to define the spectrum of progenitor cell types in the adult brain. These considerations aside, multipotential and neurogenic progenitors are abundant in the adult human white matter and are both extractable and expandable. These cells may prove to be important agents for both induction and implantation strategies of cell-based neurological therapy.

Methods

Tissue dissociation and culture. Adult subcortical white matter was surgically obtained from 21 patients, including 14 undergoing epileptic resections (age 1–50 years; 7 males and 7 females), one undergoing aneurysmal repair (69-year-old male), 2 undergoing resections of a noncontiguous dysplastic focus (20-year-old male and 36-year-old female) and 4 undergoing traumatic temporal lobe decompressions (17–67 years old; all males). Samples were obtained from patients who consented to tissue use under protocols approved by the New York Hospital–Cornell and Columbia Presbyterian Hospital Institutional Review Boards. The samples were dissected and dissociated to single-cell suspensions using papain and DNase as described[2,33,34]. The cells were then suspended in DMEM/F12/N1 with either bFGF (20 ng/ml; Sigma, St. Louis, Missouri) alone or bFGF with NT-3 (2 ng/ml; R&D Minneapolis, Minnesota) and PDGF-AA (20 ng/ml; Sigma), and plated in 100-mm suspension culture dishes (Corning, New York).

Magnetic separation of A2B5$^+$ cells. The number of viable cells was determined using calcein (Molecular Probes, Eugene, Oregon) 24–48 h after dissociation. The cells were then washed and incubated with A2B5 supernatant (clone 105; American Type Culture Collection, Manassas, Virginia) for 30–45 min at 4 °C, washed 3 times with PBS containing 0.5% BSA and 2 mM EDTA, and incubated with microbead-tagged mouse-specific rat IgM (1:4; Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C. The A2B5$^+$ cells were washed, resuspended and separated using positive selection columns, type MS$^+$ RS$^+$ or LS$^+$ VS$^+$ (magnetic cell sorting (MACS); Miltenyi Biotech). For flow cytometry of matched samples, cells were incubated in FITC-labeled mouse-specific goat IgM at 1:50 before FACS.

Transfection and sorting. Samples were transfected with pCNP2:hGFP after 2–6 d *in vitro*, using 2 μg of plasmid DNA and 10 μl of Lipofectin (Gibco, Carlsbad, California) as described[2,33,35]. Sorting for pCNP2:hGFP and A2B5 immunofluorescence was performed on a Becton-Dickinson FACS Vantage (San Diego, California), also as described[2,33,35]. Untransfected and IgM-exposed control cells were used to calibrate background; a false-positive rate of 1% was accepted as cutoff.

Generation of primary and secondary spheres. A2B5$^+$ and A2B5-depleted white-matter cells were distributed to a 24-well plate directly after sorting, at 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 cells/ml with 0.5 ml/well of DMEM/F12/N1 with bFGF/NT3/PDGF-AA. The resulting WMPC-derived neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells with trypsin and EDTA. The cells were plated at 3,000 cells/well. Three weeks later, the resultant secondary spheres were either dissociated and passaged again as tertiary spheres, or plated into 2% FBS with 20 ng/ml brain-derived neurotrophic factor on a polyornithine and fibronectin substrate and fixed 2 weeks later.

Lentiviral tagging and lineage analysis. A2B5-sorted cells were infected 2–5 d after separation with lentivirus (10^8 PFU/ml) expressing GFP under CMV promoter control and a WPRE5 post-transcriptional regulatory element[12,13]. The lentivirus was generated by co-transfecting plasmids pCMV/DR8.91, pMD.G, and pHRCMVGFPwsin into 293T cells as described[14]. A2B5-sorted cells were exposed to lentivirus for 24 h in polybrene-supplemented medium (8 μg/ml), then passaged into fresh medium in 24-well plates. GFP expression by tagged cells was observed within 2 d. The primary spheres that arose in these cultures were dissociated 3 weeks later and replated at 3,000 cells/well; secondary spheres arose from these within 2 weeks.

TRAP assay. Telomerase activity was determined using the TRAP assay[23,24], described in detail in the material accompanying Supplementary Figure 1 online.

***In utero* transplantation.** Transuterine xenograft into E17 rat fetuses was performed as described[21,36]. Some cells were injected within 24–48 h after sorting and others after 10 d *in vitro* in FGF2, PDGF-AA and NT3. One month after implantation, the animals were perfusion-fixed by 4% paraformaldehyde. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Immunocytochemistry. Xenografted rat brains were cryosectioned at 15 μm, permeabilized with PBS, 0.1% saponin and 1% NGS, and blocked with PBS, 0.05% saponin and 5% NGS, each for 30 min. Sections were labeled with HNA-specific mouse antibody (1:50; Chemicon, Temecula, California), then immunostained with βIII-tubulin–specific antibody TuJ1 (1:600; Covance, Princeton, New Jersey), MAP2-specific antibody AP-20 (1:50; Sigma), HuC/HuD-specific mouse monoclonal antibody 16A11 (25 μg/ml; H. Furneaux, Memorial Sloan-Kettering Cancer Center, New York), GAD67-specific rabbit antibody (1:100; Chemicon), GFAP-specific mouse antibody SMI 21 (1:1,000; Sternberger, Lutherville, Maryland), GFAP-specific rabbit antibody (1:400; Sigma), CNP-specific mouse antibody SMI 91 (1:1000 Sternberger), human nestin–specific rabbit antibody (1:200; Chemicon), or doublecortin-specific rabbit antisera (1:100; C. Walsh, Harvard Medical School, Boston, Massachusetts). The sections were incubated with antibody overnight at 4 °C. Species- and isotype-specific fluorescent secondary antibodies were applied at 1:100 for 1.5 h at room temperature.

O4 and A2B5 were immunolabeled *in vitro* as described[2]. For multiple-antigen labeling, O4 was localized on live cells that were then fixed and stained for βIII-tubulin, MAP2, GFAP, Hu, GAD67 or BrdU. O4 supernatant (R. Bansal and S. Pfeiffer, University of Connecticut Health Center, Farmington, Connecticut) was used at 1:100 for 40 min at 4 °C. Antibodies against βIII-tubulin, MAP-2, GFAP and BrdU (BrdU-specific rat antibody; 1:200; Harlan, Indianapolis, Indiana) were incubated overnight at 4 °C. Fixed cultures were counterstained with DAPI (10 μg/ml; Molecular Probes).

Confocal imaging. In the xenografted brains, single cells that appeared co-labeled for both human- and cell-specific markers were evaluated by confocal imaging as described[21,37]. To be deemed double labeled, cells were

required to have HNA-specific signal surrounded by neuronal or glial immunoreactivity in every serially acquired 0.4-μm *z*-dimension optical section, as well as in each orthogonal side view thereof.

Calcium imaging. Outgrowths from both first- and second-passage WMPC-derived neurospheres were assessed 2–3 weeks after plating into BDNF-supplemented DMEM/F12/N1 with 2% FBS. These mixed neuronal and glial outgrowths were challenged with 100 μM glutamate or 60 mM potassium. Cytosolic calcium imaging was conducted using confocal microscopy of cultures loaded with Fluo-3 acetoxymethylester (Molecular Probes)[33,38,39]. We previously reported that adult progenitor–derived human neurons showed a mean calcium rise of >400% in response to 60 mM potassium *in vitro*, compared with glial responses of <20%[38]. In this study, we assigned neuronal identity to cells with ≥2-fold calcium elevations to depolarization.

Electrophysiology. Sister cultures to those subjected to calcium imaging were assessed by whole-cell patch-clamp analysis. Whole-cell voltage-clamped recordings of fiber-bearing cells were conducted and analyzed as described[15,33]. A holding potential of –60 mV and voltage steps of 10 mV with 100-ms durations were applied to the recorded cells through the patch electrodes. Signals were sampled every 50 μs.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain

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Both late-gestation and adult human forebrain both contain large numbers of oligodendrocyte progenitor cells (OPCs). These cells may be identified by their A2B5$^+$PSA-NCAM$^-$ phenotype (positive for the early oligodendrocyte marker A2B5 and negative for the polysialylated neural cell adhesion molecule). We used dual-color fluorescence-activated cell sorting (FACS) to extract OPCs from 21- to 23-week-old fetal human forebrain, and A2B5 selection to extract these cells from adult white matter. When xenografted to the forebrains of newborn shiverer mice, fetal OPCs dispersed throughout the white matter and developed into oligodendrocytes and astrocytes. By 12 weeks, the host brains showed extensive myelin production, compaction and axonal myelination. Isolates of OPCs derived from adult human white matter also myelinated shiverer mouse brain, but much more rapidly than their fetal counterparts, achieving widespread and dense myelin basic protein (MBP) expression by 4 weeks after grafting. Adult OPCs generated oligodendrocytes more efficiently than fetal OPCs, and ensheathed more host axons per donor cell than fetal cells. Both fetal and adult OPCS phenotypes mediated the extensive and robust myelination of congenitally dysmyelinated host brain, although their differences suggested their use for different disease targets.

A broad range of pediatric leukodystrophies and storage diseases manifest with myelin failure or loss. Recent studies have focused on the use of transplanted oligodendrocytes or their progenitors to treat congenital myelin diseases. The myelinogenic potential of implanted brain cells was first noted in the shiverer mouse[1,2]. Shiverer is an autosomal recessive mutation; *shi/shi* homozygotes fail to develop MBP or compact myelin and die by 20–22 weeks. Transplanted fetal brain cells[3–6], primary[7] and immortalized[8] neural progenitors, and enriched glial progenitor cells[9] can all myelinate shiverer axons, albeit typically with low efficiency. Similarly, rodent subventricular zone progenitors can engraft another dysmyelinated mutant, the myelin-deficient rat, after perinatal administration[10,11]. Indeed, all of these studies suggest the feasibility of myelinating congenitally dysmyelinated brain, even though none of the cell sources used did so efficiently.

On this basis, we asked whether highly enriched populations of OPCs directly isolated from the human brain might be used as more effective substrates for cell-based therapy of congenital dysmyelination. Specifically, we postulated that human OPCs, whether derived from the fetal brain during its period of maximum oligoneogenesis, or from the adult subcortical white matter[12,13], could mediate large-scale myelination of a congenitally dysmyelinated host. We report here that both fetal and adult human OPCs, highly enriched by surface antigen–based FACS, were capable of widespread and high-efficiency myelination of the shiverer mouse brain after perinatal xenograft. We also report significant differences in the behavior of fetal and adult-derived OPCs, which suggests that they may be useful in treating different specific disease targets.

Cells dissociated from the late second-trimester human ventricular zone (21–23 weeks gestation) were first magnetically sorted to isolate A2B5$^+$ cells[13–16], including oligodendrocytic and neuronal progenitor cells. Because PSA-NCAM is expressed by immature neurons at this stage of development[17], we then used FACS to deplete PSA-NCAM$^+$ neurons from the larger A2B5$^+$ cell population. This yielded a subpopulation of A2B5$^+$PSA-NCAM$^-$ cells, which defined our oligodendrocyte progenitor pool. Two-color FACS showed that the A2B5$^+$PSA-NCAM$^-$ fraction constituted 15.4 ± 4.8% of the cells in samples from the 21- to 23-week ventricular zone (n = 5; Supplementary Figure 1 online). Of these A2B5$^+$PSA-NCAM$^-$ cells, 76.1 ± 0.5% expressed oligodendrocytic O4 by 1 week after FACS, whereas only 7.5 ± 0.3% expressed astrocytic glial fibrillary acidic protein (GFAP) and only 2.0 ± 1.3% expressed neuronal β-III tubulin. These data support the glial restriction and oligodendrocytic bias of sorted A2B5$^+$PSA-NCAM$^-$ cells. Because we achieved higher net yields with immunomagnetic separation of A2B5$^+$ cells followed by FACS depletion of NCAM$^+$ cells, compared with two-color FACS, we used this technique for progenitor isolation.

Homozygous *shi/shi* mice were injected intracallosally with fetal progenitor cell isolates on either their day of birth (P0) or on postnatal day 1 (P1), and later killed at 4, 8, 12 or 16 weeks of age. None of the animals were immunosuppressed; we relied on perinatal tolerization to ensure graft acceptance[18,19]. The injections resulted in substantial engraftment, defined as ≥100 cells per coronal section at three rostrocaudal levels sampled >100 mm apart, in 34 of the 44 neonatal mice injected for this study (25 of 33 injected with fetal human OPC, and 9 of the 11 injected with adult-derived OPCs). By 12 weeks of age, the recipients showed donor engraftment throughout the callosum and capsular and commissural

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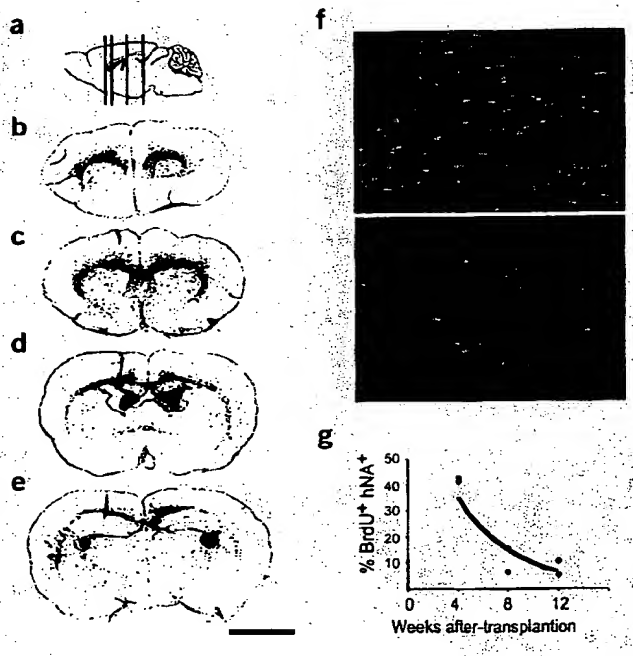

Figure 1 Fetal human OPCs disperse rapidly to infiltrate the forebrain. (a–e) Human cells were localized by immunostaining for hNA. Low-power fluorescence images of coronal section of forebrain(b–e) were collected at representative anteroposterior levels as indicated in schematic (a; ref.25). Engrafted cells are shown in red (b–e). (f) Immunofluorescent detection of BrdU (green) and hNA (red) 4 (top) and 12 (bottom) weeks after xenograft of human OPCs into shiverer mice. Arrows indicate mitotic human OPCs ($BrdU^+hNA^+$). (g) Regression plot of mitotically active donor cells as a function of time after perinatal implant. Rate of BrdU incorporation declined according to the exponential regression $y = 83.4e^{-0.22x}$, with correlation coefficient $r = -0.87$ ($P = 0.012$). Scale bar, 3 mm (b–e) or 50 μm (f).

white matter, extending caudally to the basis pontis (Fig. 1a–e). During this time, cell division among the engrafted progenitors, though initially high at 4 weeks, fell to relatively low and stable levels by 8 and 12 weeks (Fig. 1f,g). The fraction of human donor cells that incorporated BrdU during the 48 h before mice were killed dropped from 42 ± 6.1% at 4 weeks to 8.2 ± 2.4% at 12 weeks.

During this same period, many of the fetal progenitors matured into myelinogenic oligodendrocytes, as indicated by their expression of MBP. At 4 weeks, no MBP was detectable in 10 of 11 animals, despite widespread cell dispersion; scattered MBP^+ cells were noted in one mouse. At 8 weeks, patchy foci of MBP expression were noted in four of seven mice, and by 12 weeks, widespread MBP expression was noted throughout the forebrain white matter tracts in five of seven mice. By this time, the engrafted mice typically expressed MBP throughout the entire corpus callosum, as well as throughout the fimbria and internal capsules (Fig. 2a–d). Because shiverer mice express only the first exon of the *Mbp* gene[2], and hence have no immunodetectable MBP, any MBP detected in these recipients was necessarily donor-derived[8]. In addition, optical sectioning confirmed that the MBP^+ cells were of human origin, in that each MBP^+ profile was associated with a human nuclear antigen $(hNA)^+$ soma (Fig. 2c,e–h).

We next asked whether donor-derived myelin effectively wrapped host axons. We used confocal imaging and electron microscopy to assess axonal ensheathment and myelin compaction, respectively. Confocal analysis was first done on the brains of three shiverer mice that were implanted on P1 with 100,000 fetal human OPCs each, and sacrificed at 12 weeks. Foci of dense MBP expression were assessed by confocal imaging, after immunolabeling for hNA and neurofilament (NF) protein to detect donor-derived cells and host shiverer axons, respectively. We found

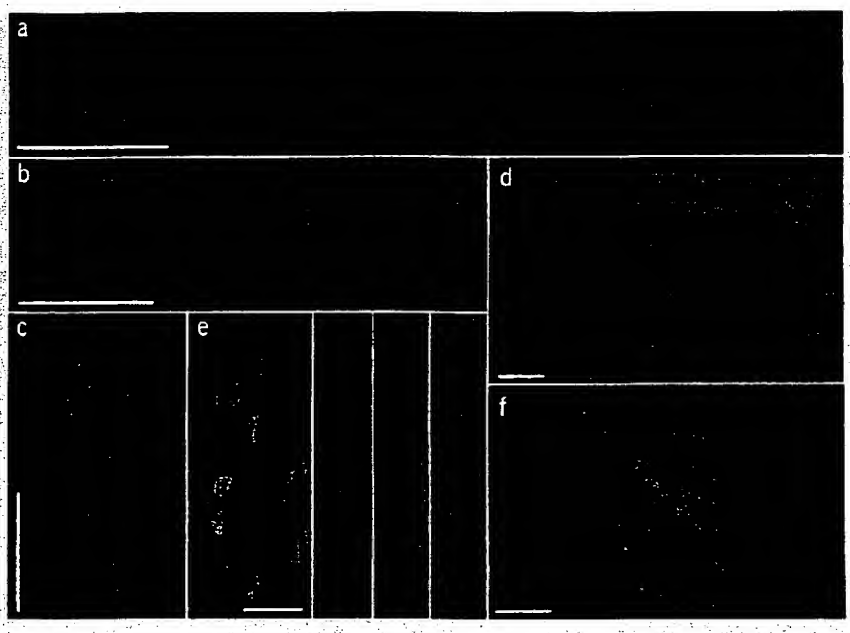

Figure 2 Engrafted human OPCs myelinate an extensive region of the forebrain. (a,b) MBP expression (green) by sorted human fetal OPCs implanted into homozygous shiverer mice. Large regions of the corpus callosum were myelinated by 12 weeks. **a** and **b** are two different mice. (c) Human OPCs migrated to and myelinated fibers throughout the dorsoventral extents of the internal capsules, resulting in widespread forebrain remyelination after a single perinatal injection. (d) MBP expression (green) in engrafted shiverer mouse callosum 3 months after perinatal xenograft was associated with human hNA^+ donor cells (red). (e) Confocal optical sections of implanted shiverer mouse callosum, with hNA^+ donor cells (red) surrounded by MBP (green). Human cells (arrows) were found within meshwork of MBP^+ fibers. Right three images, taken 1 μm apart, were merged to form left image. (f) Striatocallosal border of shiverer mouse brain, 3 months after perinatal engraftment with human fetal OPCs (blue). Donor-derived MBP^+ oligodendrocytes and myelin (red) are evident in the corpus callosum, while donor-derived $GFAP^+$ astrocytes (green) predominate on the striatal side. Scale bar, 1 mm (a–c), 100 μm (d), 20 μm (e) or 200 μm (f).

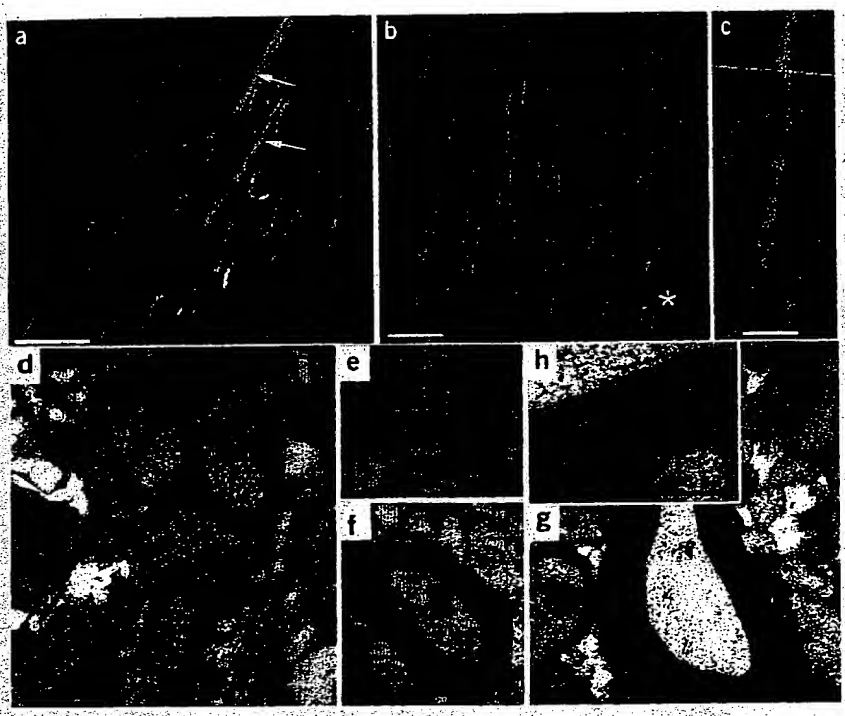

Figure 3 Axonal ensheathment and myelin compaction by engrafted human OPCs. (a) Confocal micrograph showing triple immunostain for MBP (red), human nuclear antigen (HNA; blue) and NF (green). All MBP immunostaining is derived from sorted human OPCs, whereas NF+ axons are those of mouse host. Arrows indicate segments of mouse axons ensheathed by human oligodendrocytic MBP. (b) Composite of optical sections through corpus callosum of shiverer recipient killed 12 weeks after fetal OPC implantation. (c) Higher magnification of area indicated by * in b. MBP immunoreactivity (red) surrounds ensheathed axons (green) on both sides. (d) Electron micrographs of sagittal section through corpus callosum of adult *shi/shi* homozygote. Shiverer axons typically have single loose wrapping of uncompacted myelin, such that major dense lines do not form. (e–h) Representative electron micrographs of 16-week-old homozygous shiverer mice implanted with human OPCs shortly after birth. These images show resident shiverer axons with densely compacted myelin sheaths. h, enlargement of area indicated by * in g. Major dense lines are visible between myelin lamellae, providing electron microscopic confirmation of myelination by engrafted human OPCs. Scale bar, 20 μm (a,b), 5 μm (c) or 1 μm (d–h); d, f, g use bar in e.

that the human progenitors generated myelinating oligodendrocytes in great numbers. Of the recipients scored, 11.9 ± 1.6% (mean ± s.e.m.) of NF+ host callosal axons were surrounded by MBP immunoreactivity (n = 3 mice, three fields scored per animal; Fig. 3a–c). We next used electron microscopy to verify that host axons were fully ensheathed by donor-derived oligodendrocytes, and that the latter generated compact myelin. Because MBP is required to compact consecutive layers of myelin together, its expression is required for the major dense line of mature myelin. Myelin in MBP-deficient shiverer mice did not show more than a few loose wrappings and lacked major dense lines (Fig. 3d), whereas *shi/shi* graft recipients showed compact myelin with major dense lines (Fig. 3e–h). In a sample of MBP+ fields (n = 50) derived from two mice killed 16 weeks after perinatal implant, 7.4% of callosal axons (136 of 1,832 sampled) had donor-derived myelin sheaths, as defined ultrastructurally by their major dense lines. Thus, engrafted fetal human OPCs efficiently differentiated into myelinogenic oligodendrocytes.

Some transplanted fetal OPCs differentiated into GFAP+ astrocytes as early as 4 weeks after implantation. In white-matter regions sampled on the basis of high donor-cell engraftment, 12.7 ± 4.3% of fetal donor-derived cells expressed astrocytic GFAP at 12 weeks, and 10.2 ± 4.4% of donor cells expressed MBP. No heterotopic β-III tubulin- or MAP-2-defined neurons of donor derivation were noted at 4, 8 or 12 weeks after implant (n = 33 total). Nevertheless, 40.3 ± 4.2% of donor cells expressed S100-β, which is expressed by astrocytes and young oligodendrocytes, and nestin was expressed by 47.3 ± 4.2%, suggesting that a large proportion of donor cells persisted as glial progenitors after engraftment. Fetal OPCs were recruited as oligodendrocytes or astrocytes in a context-dependent manner, giving rise to both oligodendrocytes and fibrous astrocytes in the presumptive white matter, but only to GFAP+ astrocytes in the gray matter (Fig. 2f and Supplementary Fig. 2 online).

We next asked whether adult-derived OPCs differed from their fetal counterparts with respect to their dispersal, myelinogenic capacity, or time courses thereof. We implanted two litters of P0 shiverer mice with A2B5-sorted OPCs extracted from adult human subcortical white matter. The mice were killed after 4, 8 or 12 weeks, and their brains were stained for hNA and either MBP or GFAP. Nine of 11 mice were successfully engrafted. The adult OPCs achieved widespread and dense MBP expression by 4 weeks (Fig. 4a–d); at 12 weeks, 39.5 ± 16.3% of adult OPCs expressed MBP. In contrast, none of the hNA+ fetal donor OPCs expressed MBP 4 weeks after engraftment, and only 10.2 ± 4.4% did so by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP+ cells in fetal and adult-derived grafts; Fig. 4a–c). These results indicate that engrafted adult OPCs were at least four times more likely to become oligodendrocytes and develop myelin than their fetal counterparts. Essentially no adult OPCs became astrocytes in the recipient white matter (none developed GFAP expression), whereas 12.7 ± 4.3% of fetal OPCs did so by 12 weeks. Thus, whereas nominally oligodendrocytic progenitors derived from the fetal brain acted as glial progenitors, adult OPCs behaved in a more restricted manner, largely generating either myelinogenic oligodendrocytes or persistent progenitors in recipient white matter. The more rapid myelination by adult OPCs was reflected ultrastructurally, as the major dense lines of compact myelin were readily evident in mice 6 weeks after implantation with adult OPCs at birth (Fig. 4e). No such evidence of myelin compaction was noted in mice implanted with fetal OPCs until 12–16 weeks postnatally.

Despite the apparent competitive advantage of adult OPCs, substantially more fetal than adult donor cells became engrafted in the recipient brains (Fig. 4f). At the midline of the corpus callosum, the region of maximal engraftment, we scored 1,123 ± 205.6 hNA+ fetal donor cells/mm^2. Of these, 117 ± 43.7 were MBP+, and 9.8 ± 3.1% of fetal donor cells dif-

ferentiated into myelinating oligodendrocytes by 12 weeks. In contrast, only 244 ± 182.1 donor cells/mm^2 were noted in the callosal midline of shiverer mice implanted with adult OPCs. Yet 81 ± 59.7, or 38.9 ± 12.9%, of these cells had developed into MBP$^+$ oligodendrocytes by 12 weeks ($P < 0.001$ by two-tailed t-test comparing the proportion of MBP$^+$ cells in fetal and adult grafts; Fig. 4g). In addition, whereas 12.7 ± 4.3% of fetal donor cells matured to express GFAP, no adult donor cells gave rise to GFAP$^+$ astrocytes, again suggesting a stronger bias toward the oligodendrocytic phenotype by the adult progenitors. Thus, besides maturing more quickly than fetal OPCs, adult OPCs gave rise to oligodendrocytes in much higher proportions than their fetal counterparts.

To assess whether adult and fetal OPCs differ in the extent to which they ensheath axons, we scored the numbers of axons myelinated by each donor OPC, as defined by confocal-verified MBP$^+$ wrapping of NF$^+$ axons. These absolute values were then expressed as ratios to total number of donor cells and to donor-derived MBP$^+$ oligodendrocytes per field. When assessed 12 weeks after perinatal graft, adult-derived OPCs ensheathed many more host axons per donor cell than their fetal counterparts, an effect that persisted even after we limited our analysis to the number of ensheathed axons per MBP$^+$ donor cell (Fig. 4h). In each case, the difference between fetal and adult donor ensheathment efficiency was significant by Mann-Whitney analysis ($P < 0.02$). Thus, adult-derived OPCs matured to ensheath more axons per donor cell than their fetal counterparts.

These results indicate that isolates of human OPCs sorted from the highly oligoneogenic, late second-trimester forebrain, as well as from adult subcortical white matter, can broadly myelinate the shiverer mouse brain, a genetic model of perinatal leukodystrophy. When introduced as highly enriched isolates, both fetal and adult-derived OPCs spread widely throughout the presumptive white matter, ensheathed resident mouse axons and formed antigenically and ultrastructurally compact myelin. Donor-derived myelinogenesis was geographically extensive and was observed throughout all white matter regions of the telencephalon. After implantation, the mitotic expansion of the cells slowed over time (Fig. 1g), and neither undesired phenotypes nor parenchymal aggregates were generated. Both fetal and adult-derived OPCs were capable of remyelinating mouse axons, and neither generated heterotopic neurons. We also noted some marked differences between fetal and adult-derived OPCs. Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently, and cogenerated astrocytes in recipient white matter as readily as they did myelinogenic oligodendrocytes. In contrast, adult OPCs migrated over shorter distances, but myelinated more rapidly and in higher proportions than did their fetal counterparts, with virtually no astrocytic coproduction. On an individual basis, each adult OPC–derived oligodendrocyte ensheathed and myelinated substantially more axons than did its fetal-derived counterparts (Fig. 4g).

Together, these observations suggest that isolates of human glial progenitor cells may provide effective cellular substrates for remyelinating the congenitally dysmyelinated or hypomyelinated brain. In practical terms, the choice of stage-defined cell type may be dictated by both the availability of donor material and the specific biology of the disease target. Their differences notwithstanding, fetal and adult-derived human OPC isolates were capable of achieving widespread and efficient myelination of the dysmyelinated brain, suggesting new strategies for the treatment of the congenital leukodystrophies and myelin disorders.

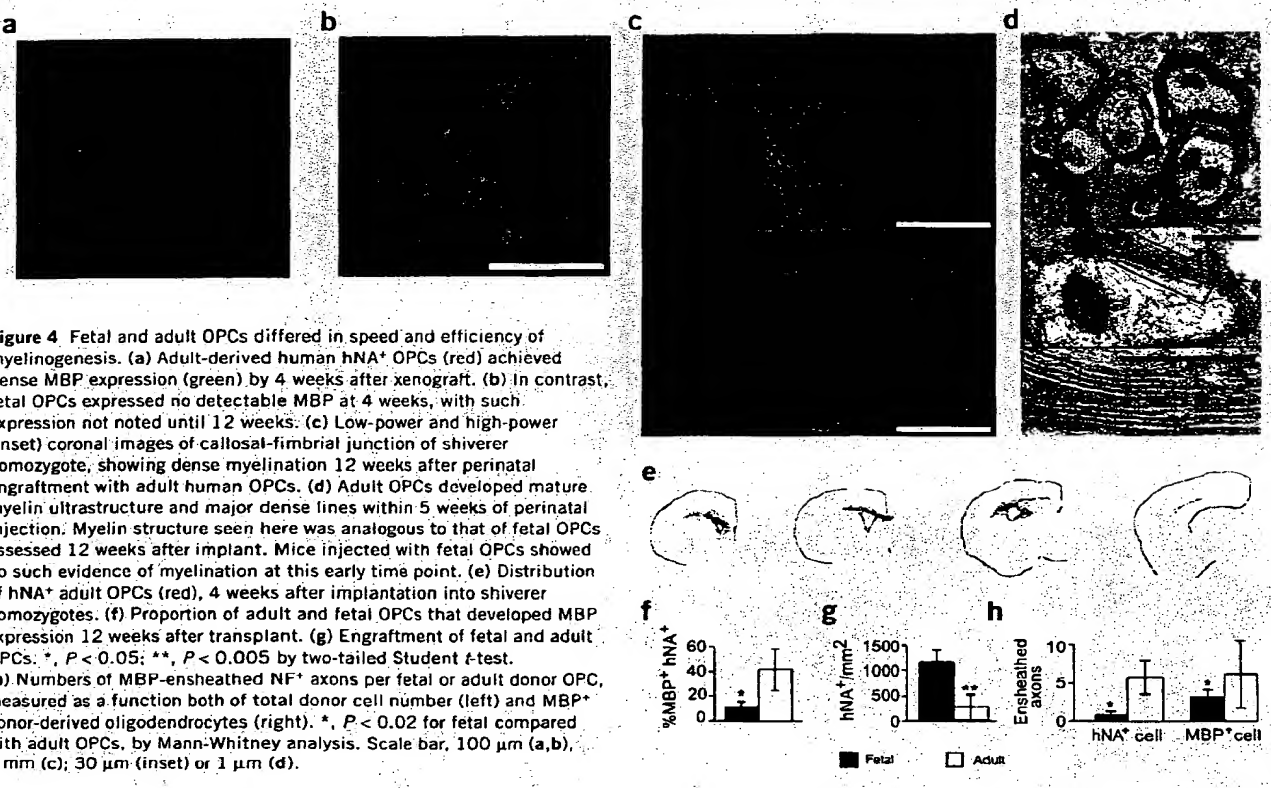

Figure 4 Fetal and adult OPCs differed in speed and efficiency of myelinogenesis. (a) Adult-derived human hNA$^+$ OPCs (red) achieved dense MBP expression (green) by 4 weeks after xenograft. (b) In contrast, fetal OPCs expressed no detectable MBP at 4 weeks, with such expression not noted until 12 weeks. (c) Low-power and high-power (inset) coronal images of callosal-fimbrial junction of shiverer homozygote, showing dense myelination 12 weeks after perinatal engraftment with adult human OPCs. (d) Adult OPCs developed mature myelin ultrastructure and major dense lines within 5 weeks of perinatal injection. Myelin structure seen here was analogous to that of fetal OPCs assessed 12 weeks after implant. Mice injected with fetal OPCs showed no such evidence of myelination at this early time point. (e) Distribution of hNA$^+$ adult OPCs (red), 4 weeks after implantation into shiverer homozygotes. (f) Proportion of adult and fetal OPCs that developed MBP expression 12 weeks after transplant. (g) Engraftment of fetal and adult OPCs. *, $P < 0.05$; **, $P < 0.005$ by two-tailed Student t-test. (h) Numbers of MBP-ensheathed NF$^+$ axons per fetal or adult donor OPC, measured as a function both of total donor cell number (left) and MBP$^+$ donor-derived oligodendrocytes (right). *, $P < 0.02$ for fetal compared with adult OPCs, by Mann-Whitney analysis. Scale bar, 100 μm (a,b), 1 mm (c); 30 μm (inset) or 1 μm (d).

METHODS

Cells. Fetal OPCs were extracted from 21- to 23-week-old human fetuses obtained at abortion. The forebrain ventricular and subventricular zones were dissected free and chilled on ice. The minced samples were dissociated using papain and DNase as described[20,21], always within 3 h of extraction, and maintained overnight in DMEM/F12/N1 with 20 ng/ml fibroblast growth factor. Adult-derived OPCs were collected from subcortical white matter samples obtained at surgery, as described[12,13]. The eight adult tissue samples used were derived largely from patients undergoing temporal lobe resection for medication-refractory epilepsy. No tissues were accepted from patients with known neoplastic disease. Both fetal and adult samples were obtained with consent, using protocols approved by the institutional review boards of Cornell–New York Presbyterian Hospital, and the Albert Einstein College of Medicine and Jacobi Hospital.

Sorting. The day after dissociation, cells from fetal samples were incubated in a 1:1 ratio with monoclonal antibody A2B5 supernatant (clone 105, American Type Culture Collection) for 30 min, then washed and labeled with fluorophore- or microbead-tagged rat antibody to mouse IgM (Miltenyi Biotec). In some instances, two-channel FACS was used to define the proportions and homogeneity of A2B5- and PSA-NCAM-defined subpopulations, using a FACSVantage SE/Turbo (Becton Dickinson) as described[13,21]. For preparative sorting before transplantation, $A2B5^+$ cells were prepared by magnetic separation (Miltenyi Biotec) according to the manufacturer's protocol. The bound cells were eluted and incubated with mouse antibody to PSA-NCAM (1:25; PharMingen) for 30 min, then with phycoerythrin-tagged secondary antibody (1:200). The PSA-$NCAM^+$ population was then removed by FACS, leaving a highly enriched pool of $A2B5^+PSA\text{-}NCAM^-$ cells. This PSA-NCAM immunodepletion step was omitted for adult samples, which were sorted on the basis of A2B5 only[12,20]. After sorting, both fetal and adult cells were maintained for 1–7 d in DMEM/F12/N1 with 20 ng/ml basic fibroblast growth factor (20 ng/ml) until implantation.

Transplantation and tagging. Homozygous shiverer mice were bred in our colony. Within 1 d of birth, pups were cryoanesthetized for cell delivery. Donor cells (1×10^5) in 2 µl of HBSS were injected through a pulled glass pipette and inserted through the skull into the presumptive corpus callosum. Transplants were directed to the corpus callosum at a depth of 1.0–1.2 mm, depending on the weight of the pup, which varied from 1.0 to 1.5 g. Pups were killed 4, 8, 12 or 16 weeks thereafter. For some experiments, recipient mice were injected for with bromodeoxyuridine (BrdU; 100 µg/g as a 1.5 mg/100 µl solution) every 12 hours 2 d before killing.

Immunohistochemistry. Transplanted cells were identified using antibody 1281 to human nuclei (Chemicon), monoclonal antibody 91 to cyclic nucleotide phosphodiesterase (CNP) protein (Sternberger and Meyer), rabbit antibody to S-100 (Sigma), rabbit antibody to human nestin (gift of H. Okano, Keio University), Sternberger monoclonal antibody 311 to NF, Sternberger monoclonal antibody 21 to human GFAP, rat antibody to BrdU (Harlan) and either Sternberger monoclonal antibody 94 to MBP or rat antibody 7349 to MBP (Abcam), all as described[7,12,20–22].

Confocal and electron microscopy. Confocal imaging was done using an Olympus Fluoview mated to an IX70 inverted microscope, as described[23]. Argon laser lines were used to achieve three-channel immunofluorescence detection of fluorescein-, Texas red– and Cy5-tagged antibodies; the latter was then pseudocolored blue for presentation. For confocal quantification of ensheathment efficacy, shiverer axons were scored as ensheathed when yellow index lines intersected NF^+ axon abutted on each side by MBP immunoreactivity. The proportion of ensheathed axons was defined as the incidence of MBP^+NF^+ axons divided by the total number of NF^+ axons in each field. For electron microscopy, animals were perfused and post-fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 6% sucrose, then Vibratome-sectioned as alternating thick (400 µm) and thin (100 µm) sections. The latter were immunostained for MBP. Thick sections adjacent to thin sections with MBP expression were then processed in 1% osmium and 1.5% ferricyanide, stained with 1.5% uranyl acetate, embedded in Epon, cut as 100-nm thin sections onto Formvar-coated grids, stained with lead citrate and visualized using a JEOL100 electron microscope[24].

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Medicine website for details).

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A stochastic model of brain cell differentiation in tissue culture

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Abstract. The timing of cell differentiation can be controlled both by cell-intrinsic mechanisms and by cell-extrinsic signals. Oligodendrocyte type-2 astrocyte progenitor cells are known to be the precursor cells that give rise to oligodendrocytes. When stimulated to divide by purified cortical astrocytes or by platelet-derived growth factor, these progenitor cells generate oligodendrocytes *in vitro* with a timing like that observed *in vivo*. The most widely accepted model of this process assumes a cell-intrinsic biological clock that resides in the progenitor cell. The intrinsic clock model originally proposed in 1986 remains as the dominant theoretical concept for the analysis of timed differentiation in this cell lineage. However, the results of a recent experimental study (Ibarrola et al., Developmental Biology, vol. 180, 1–21, 1996) are most consistent with the hypothesis that the propensity of a clone of dividing O-2A progenitor cells initially to generate at least one oligodendrocyte may be regulated by cell-intrinsic mechanisms, but that environmental signals regulate the extent of further oligodendrocyte generation. We propose a stochastic model of cell differentiation in culture to accommodate the most recent experimental findings. Our model is an age-dependent branching stochastic process with two types of cells. The model makes it possible to derive analytical expressions for the expected number of progenitor cells and of oligodendrocytes as functions of time. The model parameters were estimated by fitting these functions through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. Using this method we provide a biologically meaningful interpretation of the observed pattern of oligodendrocyte generation *in vitro* and its modification in the presence of thyroid hormone.

Key words: Brain cells – Differentiation – Proliferation – Branching process

1 Introduction

It is a striking feature of ontogenic development that particular cell types first appear at precisely regulated moments in the history of the organism and then increase in number over a time period that is very similar in members of the same species. Understanding the biological principles which underly such appropriately timed cell generation is one of the profound challenges in developmental biology.

Any analysis of the timely generation of differentiated cell types must by necessity begin with investigation of the transition from dividing precursor cell to differentiated (and often non-dividing) progeny cell. In order to obtain data that allow one to develop an understanding of the complexities of such differentiation processes, it is necessary to gain access to a wide range of detailed information at a clonal level. Ideally, one should be able to examine multiple clones of dividing precursor cells and to unambiguously distinguish between precursor cells and differentiated progeny. If one is interested in analysis of these processes in cells derived from vertebrates, then experimental studies of this nature must be performed in tissue culture (to enable visualization of cell clones and their development over extended time periods), leading to the further requirement that it be possible to mimic *in vitro* the developmental processes which are thought to occur *in vivo*.

One of the few cellular lineages in which it is possible to obtain the complete collection of data described above is derived from the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell of the central nervous system. It is possible to grow O-2A progenitors cells *in vitro* in such a manner that they divide and generate oligodendrocytes with a timing which recapitulates the timing of normal development *in vivo*. O-2A progenitor cell division can be promoted by purified cortical astrocytes (of a separate glial lineage), and this induction of cell division can also be brought about by platelet-derived growth factor (PDGF) and by the O-2A progenitor mitogen produced by cortical astrocytes (Noble and Murray, 1984; Noble et al., 1988; Pringle et al., 1991; Raff et al., 1988; Bogler et al., 1990). Embryo-derived O-2A progenitor cells induced to divide by PDGF or by type-1 astrocytes will generate oligodendrocytes with a timing that mimics *in vitro* the timing which is seen *in vivo* (Raff et al., 1988).

The initiation of the timely generation of oligodendrocytes within a clonal family of dividing O-2A progenitor cells has for many years been thought to be controlled by a cell-intrinsic biological clock that induces symmetric and synchronous differentiation of all clonally-related O-2A progenitor cells into oligodendrocytes within a relatively short period of time (Temple and Raff, 1985). According to this model, oligodendrocyte generation is associated with preclusion of the self-renewal process of precursor cells, and thus is in some ways analogous to the limited mitotic life-span expressed by many primary cell types. This hypothesis provided a simple model for the study of temporally regulated differentiation, but it now seems likely that this hypothesis is both incorrect and overly simplistic.

In contrast to the above hypothesis, it was recently discovered that instead of differentiating symmetrically, clonal families of O-2A progenitor may undergo a protracted period of oligodendrocyte generation, during which time the probability of precursor cell self-renewal is regulated predominantly by cell-extrinsic signaling molecules rather than by cell-intrinsic biological clocks (Ibarrola et al., 1996). In other words, it was found that it is possible to distinguish experimentally between the probability that a clone of dividing O-2A progenitor cells will generate at least one oligodendrocyte at an appropriate time *in vitro* and the actual extent of oligodendrocyte generation in that clone. While the latter process was influenced by the presence or absence of exogenous factors (such as thyroid hormone), the former was not.

In respect to the initial generation of oligodendrocytes, it appears that the extent of oligodendrocyte generation within an individual clone may be stochastic. For example, there was no apparent relationship between the number of oligodendrocytes found and the ratio of oligodendrocytes to progenitor cells, excepting that the rare colonies consisting wholly of oligodendrocytes all contained 10 cells or less. In addition, when oligodendrocytes first appeared in cultures of embryonic brain cells the number of oligodendrocytes per colony ranged from 1 to 88, the proportion of oligodendrocytes in heterogeneous colonies ranged from <1% to 81%, and the fractional representation of oligodendrocytes in a colony was not correlated with the number of oligodendrocytes in that colony.

In this paper, we develop a stochastic model which is specifically designed to make quantitative inferences from experimental data on brain cell differentiation *in vitro*.

2 Experimental procedures

Purification of O-2A progenitor cells is described at length by Ibarrola et al. (1996). Purified progenitor cells derived from 7 day old animals were plated at a density of 2500-3000 cells. Cells were fed with PDGF. After plating the culture was scored for the presence of individual cells. Plates with cells in clumps were discarded and not included in the experiment. A fixed number of clones was randomly selected and the cell type composition of each clone was recorded at different times. Cell-types were identified by morphology and cell types were confirmed at the end of each experiment by immunofluorescence using cell type specific antibodies. The design of experiments with thyroid hormone was identical.

3 The model for oligodendrocyte generation in culture

Our model for the stochastic mechanism of O-2A progenitor cell differentiation *in vitro* is a multitype age-dependent branching stochastic process. The model structure is defined by the following set of assumptions.

(i) The process begins with a single progenitor, or type-1, cell cultured at time $t = 0$. This initiator cell may, when it divides, produce two types of cells: progenitor cells of the same type, and oligodendrocytes or type-2 cells. The initiator cell and its descendants are not susceptible to death.

(ii) At the end of the mitotic cycle, every type-1 cell gives rise to two daughter progenitor cells with probability p, and it transforms (differentiates) into an oligodendrocyte with probability $1 - p$. Thus, the probability generating function of the cell progeny is specified as

$$h_1(s) = ps_1^2 + (1 - p)s_2 , \tag{1}$$

where $s = (s_1, s_2)$. The corresponding generating function for type-2 cells is

$$h_2(s) = s_2 , \tag{2}$$

that is, oligodendrocytes neither divide nor die. We do not provide a description of cell death, lest the model be nonidentifiable. The event of cell death is very rare during the first 6 days of observation (Ibarrola et al., 1996), and we take advantage of this fact when estimating the model parameters. The death of oligodendrocytes normally begins on day 7 and its rate increases with time. The contribution of cell death at these late times can be inferred from experimental data by the indirect route.

(iii) The lengths of the mitotic cycle of the initiator cell and its descendants of the same type are independent and identically distributed nonnegative random variables with a common cumulative distribution function $F(t)$. We assume that $F(0) = 0$.

(iv) Progenitor cells, the only migratory cells in the population, do not migrate out of the field of observation.

(v) The usual independence assumptions regarding the evolution of age-dependent branching processes (Jagers, 1975) are adopted.

The model thus specified is a special case of the Bellman-Harris branching process with two types of particles; its asymptotic properties were studied in detail by Jagers (1969, 1975).

Let $Z_i(t)$, $i = 1, 2$, be the number of cells of the ith type at time t. The probability generating functions of $Z_1(t)$ and $Z_2(t)$ can be obtained using general methods of the theory of branching processes. A detailed exposition of these methods can be found in Yakovlev and Yanev (1989). Consider the two-dimentional stochastic process $Z(t) = (Z_1(t), Z_2(t))$ and introduce the generating function

$$\Phi(t, s) = (\Phi_1(t, s), \Phi_2(t, s)) , \tag{3}$$

with the components

$$\Phi_i(t, s) = \sum_k \Pr\{Z(t) = k | Z(0) = e_i\} s^k , \quad i = 1, 2, |s| \leq 1 , \tag{4}$$

where $e_1 = (1, 0)$, $e_2 = (0, 1)$, and the summation in (4) is over the set of all points in R^2 with nonnegative integer coordinates. With $h_1(s)$ and $h_2(s)$ given by (1) and (2), the generating functions $\Phi(t, s)$ satisfy the following equations

$$\Phi_1(t, s) = s_1[1 - F(t)] + p\int_0^t \Phi_1^2(t - u, s)\, dF(u) + (1 - p)\int_0^t \Phi_2(t - u, s)\, dF(u)\,, \tag{5}$$

$$\Phi_2(t, s) = s_2\,.$$

Setting $s_1 = z$ and $s_2 = 1$ in (5), it is easy to derive equations for the corresponding marginal generating functions $\varphi_1(t, z) = \Phi_1(t, z, 1)$ and $\varphi_2(t, z) = \Phi_2(t, z, 1)$:

$$\varphi_1(t, z) = z[1 - F(t)] + (1 - p)F(t) + p\int_0^t \varphi_1^2(t - u, z)\, dF(u) \tag{6}$$
$$\varphi_2(t, z) = 1\,.$$

In a similar manner, for $\psi_1(t, z) = \Phi_1(t, 1, z)$ and $\psi_2(t, z) = \Phi_2(t, 1, z)$ we obtain

$$\psi_1(t, z) = 1 - F(t) + p\int_0^t \psi_1^2(t - u, z)\, dF(u) + (1 - p)zF(t) \tag{7}$$
$$\psi_2(t; z) = z\,.$$

Let $M_1(t) = \varphi_1'(t, 1)$ be the expected number of type-1 cells at time t. From (6) it follows that

$$M_1(t) = 1 - F(t) + 2p\int_0^t M_1(t - u)\, dF(u)\,. \tag{8}$$

The expected number of type-2 cells is $M_2(t) = \psi_1'(t, 1)$. Thus we see from (7) that

$$M_2(t) = (1 - p)F(t) + 2p\int_0^t M_2(t - u)\, dF(u)\,. \tag{9}$$

The integral equations (8) and (9) can be generalized to incorporate possible death of type-1 cells (Jagers, 1975). Furthermore they can be solved in closed form (see Athreya and Ney, 1972). Introducing the notation

$$G^{*0}(t) = 1, \qquad G^{*1}(t) = G(t), \qquad G^{*(n+1)}(t) = \int_0^t G^{*n}(t - u)\, dG(u)\,,$$

the solution of (8) is represented as

$$M_1(t) = \sum_{n=0}^{\infty} (2p)^n[F^{*n}(t) - F^{*(n+1)}(t)]\,. \tag{10}$$

Similarly the solution of (9) is given by

$$M_2(t) = (1 - p)\sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t)\,. \tag{11}$$

The n-fold convolution F^{*n} of F with itself can be found in an explicit form for some distributions of the mitotic cycle duration. The most popular choice in cell-kinetics studies is the two-parameter gamma distribution (Yakovlev et al., 1977; Nedelman and Rubinow, 1981; Nedelman et al., 1987; Yakovlev and Yanev, 1989, to name a few). We shall proceed from the same choice because this parametric family is quite flexible and reflects a multistage structure of the cell cycle. Some authors (Hartmann et al., 1975; Yakovlev and Zorin, 1988) indicate that the results of kinetic analysis of cell proliferation are usually insensitive to the form of $F(t)$ where absolutely continuous unimodal distributions are concerned.

Suppose that $F(t)$ is specified as the gamma distribution with shape parameter α and scale parameter β. Then we have

$$F^{*n}(t) = \frac{\beta^{\alpha n}}{\Gamma(\alpha n)} \int_0^t x^{\alpha n - 1} e^{-\beta x} dx . \tag{12}$$

To ensure computationally convenient formulas for $M_1(t)$ and $M_2(t)$ it is reasonable to limit possible values of α to the set of positive integers. This constraint has little effect on the accuracy of estimation of the parameter α from experimental data on the mean size of cell clones. For integer α formula (12) reduces to

$$F^{*n}(t) = 1 - e^{-\beta t} \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} , \tag{13}$$

and we have

$$M_1(t) = e^{-\beta t} \left\{ \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} + \sum_{n=1}^{\infty} (2p)^n \left[\sum_{k=0}^{\alpha(n+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} \right] \right\} , \tag{14}$$

$$M_2(t) = (1 - p) \left[\frac{1}{1 - 2p} - e^{-\beta t} \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} - e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{\alpha(n+1)-1} \frac{(\beta t)^k}{k!} \right] . \tag{15}$$

The timing of oligodendrocyte generation *in vitro* has been found to be fundamentally similar to that which occurs *in vivo*. Therefore, it is natural to assume that the population of progenitor cells eventually becomes extinct. In terms of our model this means that the process $Z_1(t)$ is subcritical and we should limit our consideration to the case $p < 0.5$. Then formula (11) implies that $M_2(t)$ is a monotone nondecreasing function and

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = \frac{1 - p}{1 - 2p} . \tag{16}$$

The behavior of $M_2(t)$ is intuitively appealing because in the long run the population of oligodendrocytes is expected to level off to a constant size.

Difficulties emerge when we look more closely at the expected number of progenitor cells. Recalling formula (10) we see that

$$M_1(0) = 1, \qquad \lim_{t \to \infty} M_1(t) = 0 . \tag{17}$$

It also follows from (10) that $M_1(t)$ is a nonincreasing function of time. Indeed, representing the series (10) as

$$\begin{aligned} M_1(t) &= \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - \sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t) \\ &= \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - F(t) - \sum_{k=0}^{\infty} (2p)^{k+1} F^{*(k+2)}(t) \\ &= 1 - (1-2p)F(t) + \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) - \frac{1}{2p} \sum_{k=0}^{\infty} (2p)^{k+2} F^{*(k+2)}(t) \\ &= 1 - (1-2p)\left[F(t) + \frac{1}{2p} \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) \right], \end{aligned}$$

it is easy to see that $M_1(t)$ is nonincreasing in t whenever $p < 0.5$.

The behavior of $M_1(t)$ appears to be in conflict with experimental data presented in Sect. 4. Since each colony begins with exactly one clonogenic cell at time $t = 0$ one should expect (see Figs. 1 and 2, Sect. 4) that the growth curve for progenitor cells passes through a maximum before it starts decreasing; this is the only pattern consistent with our observations. Clearly, the model must be generalized to allow for the initial increase in the mean number of type-1 cells. The way to do this is through assigning a higher (greater than 0.5) probability of progenitor cell division to initial mitotic cycles. To keep the number of unknown parameters to a minimum, we assume that $p = 1$ for the first N cycles and $p < 0.5$ for the subsequent mitotic cycles. In other words, a progenitor cell acquires the competence for differentiation only after it undergoes a critical number of mitotic divisions. The parameter N is to be estimated from experimental data.

The stepwise change of the probability p can be readily incorporated into the model by introducing N additional types of cells that correspond to the N initial mitotic cycles. We will omit the derivation of the expressions for $M_1(t)$ and $M_2(t)$ given below because it parallels that of formulas (14) and (15). The functions $M_1(t)$ and $M_2(t)$ are given by

$$\begin{aligned} M_1(t) = e^{-\beta t}\Bigg\{ &\sum_{k=0}^{\alpha-1} \frac{(\beta t)^k}{k!} + \sum_{j=1}^{N-1} 2^j \left(\sum_{k=0}^{\alpha(j+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha j-1} \frac{(\beta t)^k}{k!} \right) \\ &+ 2^N \Bigg[\sum_{k=0}^{\alpha(N+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha N-1} \frac{(\beta t)^k}{k!} \\ &+ \sum_{n=1}^{\infty} (2p)^n \left(\sum_{k=0}^{\alpha(N+n+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha(N+n)-1} \frac{(\beta t)^k}{k!} \right) \Bigg] \Bigg\}, \end{aligned} \tag{18}$$

$$\begin{aligned} M_2(t) = 2^N(1-p)\Bigg[&\frac{1}{1-2p} - e^{-\beta t} \sum_{k=0}^{\alpha(N+1)-1} \frac{(\beta t)^k}{k!} \\ &- e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{\alpha(N+n+1)-1} \frac{(\beta t)^k}{k!} \Bigg], \end{aligned} \tag{19}$$

where $N = 1, 2, \ldots, \sum_{j=1}^{0} x_i = 0$, and $0 < p < 0.5$. It immediately follows from (18) and (19) that $M_1(t)$ still satisfies (17) while $M_2(t)$ has the following properties:

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = 2^N \frac{1-p}{1-2p}. \tag{20}$$

4 Inference from experimental data

Equations (19) and (20) provide the basis for estimation of the model parameters by fitting the functions $M_1(t)$ and $M_2(t)$ through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. We used the least squares method for this purpose. To minimize the sum of squared residuals, use was made of the flexible simplex method (Himmelblau, 1972). The results produced by this nonlinear programming procedure were verified with a program for function minimization included in *MATHEMATICA*.

At a time when we conducted our analysis only the data for $t = 72, 96, 120$ and 144 hours were available, furnishing an opportunity to test the predictive power of the model. In what follows we will refer to these data as Experiment 1. Using the data from Experiment 1 we obtained the following estimates of the model parameters: $\hat{p} = 0.461$, $\hat{N} = 2$, $\hat{\alpha} = 3$, $\hat{\beta} = 0.107$. Thus, the mean, $\tau = \alpha/\beta$, and the standard deviation, $\sigma = \sqrt{\alpha}/\beta$, of the mitotic cycle duration are estimated as $\hat{\tau} = 28$ h and $\hat{\sigma} = 16.2$ h, respectively. The resultant fit is shown in Fig. 1. The model predicts that the mean number of oligodendrocytes tends to a constant level of 27.6 (see formula (20)). Unfortunately, testing this prediction cannot be carried out without the addition of oligodendrocyte survival factors (Noble, 1997), all of which are known to themselves affect the probability of cell differentiation. In our experiments, the cells do not survive that long.

A similar analysis of oligodendrocyte generation in the presence of thyroid hormone (Fig. 2) resulted in the following estimates: $\hat{p} = 0.287$, $\hat{N} = 2$, $\hat{\alpha} = 3$, $\hat{\beta} = 0.137$, whence we have $\hat{\tau} = 21.3$ h and $\hat{\sigma} = 12.6$ h. Hence, thyroid hormone exerts a twofold effect on the cell system under study: it reduces the mean duration of the mitotic cycle of progenitor cells, and it increases the probability of their transformation into oligodendrocytes. The observed dynamics of accumulation of oligodendrocytes is attributable to these mechanisms. It is seen from Fig. 2 that in the presence of thyroid hormone the mean size of the population of oligodendrocytes grows more rapidly attaining a much lower constant level of 6.7 cells predicted by formula (20). At the same time the critical number of initial cycles, N, remains unaltered. These results are in full agreement with qualitative conclusions made by Ibarrola et al. (1996).

An independent experimental study (Experiment 2) was conducted to test the model behavior beyond the observation period chosen in Experiment 1. In

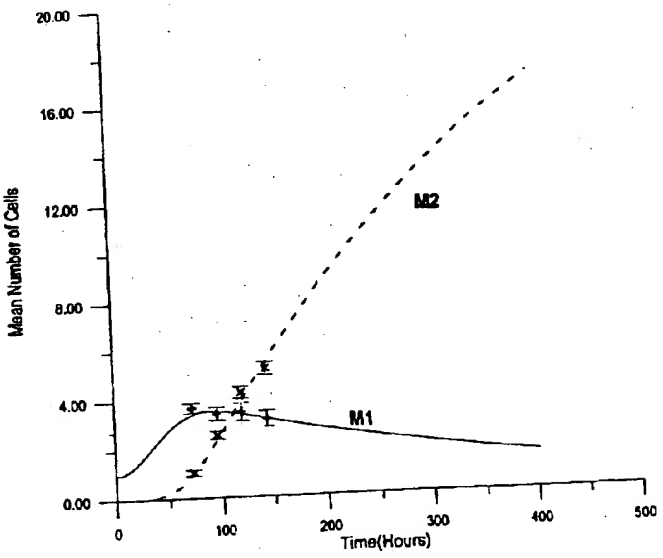

Fig. 1. The dynamic behavior of cell populations in cultures without thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time; dashed line represents the expected number of oligodendrocytes; + and × are the corresponding experimental data. Bars represent one root square error of estimation at each time point

Experiment 2, data were recorded at $t = 48$, 144 and 192 hours and compared with the corresponding values of $M_1(t)$ and $M_2(t)$ resulted from the analysis of Experiment 1. These results are summarized in Tables 1–4. It is clear from these tables that the model provides a good description of Experiment 2 except the mean number of oligodedrocytes at 192 h (Tables 2 and 4). The observed discrepancy can be attributed to a high rate of cell death at this late time which is not incorporated into the model. As our independent observations show, the proportion of oligodendrocytes surviving by day 8 under *in vitro* conditions is no more than 80%. Yet another factor that may contribute to the discrepancy under discussion is the inter-experimental variation which is difficult to control because the experiments of this kind are very time consuming. One can see from the data for $t = 144$ h that the mean number of oligodendrocyes tends to be smaller in Experiment 2 than in Experiment 1. This tendency may be a manifestation of the higher rate of cell death in Experiment 2 indicated above. However, there is little point in incorporating an explicit description of the process of cell death into the model because the

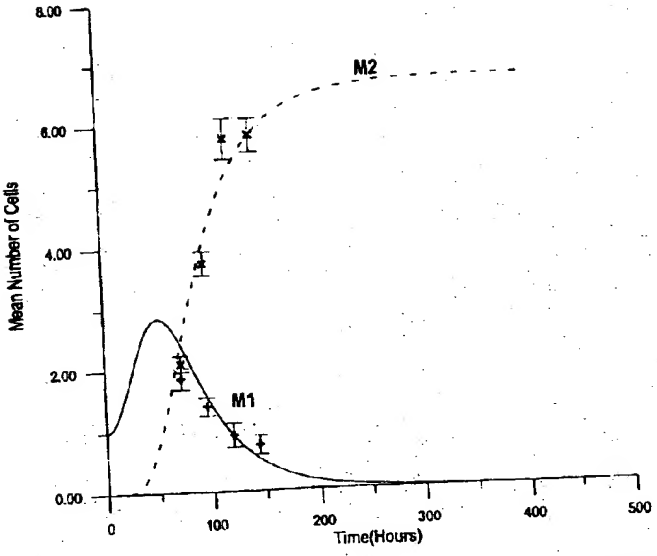

Fig. 2. The dynamic behavior of cell populations in cultures with added thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time; dashed line represents the expected number of oligodendrocytes; + and × are the corresponding experimental data. Bars represent one root square error at each time point

Table 1. Mean number of O-2A progenitor cells in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	2.68	–	3.4	–	0.24
72	3.40	3.67	–	0.20	–
96	3.54	3.44	–	0.26	–
120	3.40	3.49	–	0.37	–
144	3.20	3.19	2.02	0.33	0.27
192	2.79	–	1.65	–	0.34

available experimental data do not provide sufficient information to identify its quantitative characteristics. The observed overall agreement between the model predictions and experimental data indicates that the postulated stochastic mechanism of oligodendrocyte generation *in vitro* is biologically plausible.

Table 2. Mean number of oligodendrocytes in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	0.18	–	0.27	–	0.08
72	1.00	1.05	–	0.12	–
96	2.38	2.57	–	0.16	–
120	3.95	4.26	–	0.24	–
144	5.50	5.21	2.69	0.25	0.33
192	8.33	–	4.37	–	0.36

Table 3. Mean number of O-2A progenitor cells in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	2.83	–	2.29	–	0.19
72	2.41	1.84	–	0.17	–
96	1.55	1.39	–	0.14	–
120	0.91	0.92	–	0.19	–
144	0.53	0.76	0.92	0.15	0.22
192	0.17	–	0.56	–	0.21

Table 4. Mean number of oligodendrocytes in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	0.68	–	0.54	–	0.10
72	2.41	2.09	–	0.13	–
96	4.06	3.73	–	0.19	–
120	5.16	5.75	–	0.34	
144	5.81	5.81	3.68	0.28	0.29
192	6.40	–	5.42	–	0.37

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Measurement of Time in Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors Is a Cellular Process Distinct from Differentiation or Division

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When stimulated by platelet-derived growth factor (PDGF), oligodendrocyte-type-2 astrocyte (O-2A) progenitors derived from perinatal rat optic nerves undergo a limited number of cell divisions before clonally related cells synchronously and symmetrically differentiate into nondividing oligodendrocytes. The duration of this mitotic period is thought to be controlled by a cell-intrinsic biological clock. Thus, in the presence of PDGF, the measurement of time by the biological clock is intimately coupled to the control of division and differentiation. In contrast, O-2A progenitors grown in the presence of PDGF plus basic fibroblast growth factor (bFGF) divide indefinitely in the absence of differentiation and so do not exhibit a limited period of division. We have tested whether growth in PDGF plus bFGF alters the duration of the limited period of division O-2A progenitors exhibit in response to PDGF alone. Accordingly, O-2A progenitors were grown in the presence of PDGF plus bFGF for varying lengths of time, before being switched to conditions that promote timed differentiation (PDGF but not bFGF). Increasing duration of culture in PDGF plus bFGF led to a gradual shortening of the period for which O-2A progenitors were subsequently responsive to PDGF alone, until eventually all cells differentiated without dividing after switching. In contrast, a short exposure to bFGF was not sufficient to cause a similar alteration in the pattern of differentiation. These results indicate that O-2A progenitors prevented from undergoing timed differentiation nevertheless retain the ability to measure elapsed time, implying that the biological clock in this cell type can be uncoupled from differentiation. Furthermore, they demonstrate that the biological clock does not impose an absolute limit on the number of divisions that an O-2A progenitor can undergo. In contrast with existing hypotheses, our observations suggest that the molecular mechanism that controls timed differentiation must consist of at least two components, with the clock itself being in some manner distinct from mechanisms that limit cell division and/or directly regulate differentiation.

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INTRODUCTION

Several observations in a variety of cell types suggest that measurement of elapsed time by cells is closely linked to the initiation of terminal differentiation. For example, hematopoietic stem cells generate erythroid cells which switch from production of fetal hemoglobin to adult hemoglobin after the passage of a seemingly preprogrammed length of time (Zanjani *et al.*, 1979; Wood *et al.*, 1985). Similarly, the number of divisions a fibroblast can undergo before terminally differentiating into a senescent cell appears to be preprogrammed or limited (reviewed in Goldstein, 1990). The measurement of elapsed time also seems to play a major controlling role in the timing of differentiation of glial precursor cells of the central nervous system (CNS)[2] into oligodendrocytes (Abney *et al.*, 1981; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988; reviewed in Groves *et al.*, 1991, and Noble, 1991). The apparent coupling of the measurement of elapsed time to differentiation in these systems raises the question of whether these two processes are mechanistically distinct.

Understanding the regulatory mechanisms that make it possible for cells to differentiate according to an intrinsic schedule has been a subject of considerable inter-

[2] Abbreviations used: Ast, astrocyte coculture; Ast/P, astrocyte coculture with additional exogenous PDGF; Ast/PF, astrocyte coculture with exogenous PDGF plus bFGF; bFGF, basic fibroblast growth factor; CNS, central nervous system; DMEM-BS, Dulbecco's Modified Eagle's Medium modified according to Bottenstein and Sato, 1979; DMEM-FCS, DMEM containing fetal calf serum; E19, Embryonic Day 19; ECM, extracellular matrix; O-2A, oligodendrocyte-type-2 astrocyte; P7, Postnatal Day 7; PDGF, platelet-derived growth factor; SV40 T, simian virus 40 large tumor antigen.

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est. Hypotheses about the nature of cellular timing mechanisms are as varied as the terms used to describe the phenomenon itself. Thus the terms "biological clock," "developmental clock," "time-measuring ability of cells," "finite mitotic life span," and other similar phrases have been used to describe phenomena in which a cell-intrinsic timing or measuring system appears to control the timing of differentiation. Due to the lack of knowledge about what is being measured by biological clocks, we describe this phenomenon as the measurement of elapsed time (see for example Orgel, 1973). This should be taken only as indicating that the passage of time is associated with the changes in the behavior of cells and is not meant to suggest that cells measure time in the same way that mechanical clocks do. It has been suggested, for example, that "biological time [is] equivalent to trains of specific physical or chemical events, which is a very different concept than that of an intrinsic clock based on sidereal or calendar time" (Finch, 1990). In the instances of particular interest here, it is clear that the duration of the period to be measured is determined at least several (and sometimes many) cell divisions before the differentiation event is itself observable. Most investigations of this phenomenon have been carried out on fibroblasts (which enter a nondividing senescent state after a limited number of cell divisions) and the range of hypotheses advanced to explain the limited mitotic life span of these cells includes random accumulation of cellular damage (Szilard, 1959; Orgel, 1973; Goldstein, 1990), telomere shortening (Harley *et al.*, 1990), changes in negative-growth regulatory genes (Weinberg, 1993), and progression through a genetic program (Orgel, 1973; Bayreuther *et al.*, 1988a,b; Goldstein, 1990). One could equally imagine that timed differentiation is caused by a steady increase, over a number of cell divisions, in the amount of a differentiation-inducing activity which determines cellular phenotype only after passing a threshold. Alternately, the steady decrease in the amount of some activity absolutely required for cell division could trigger the timed cessation of division.

All of the hypothetical mechanisms proposed to explain the workings of biological clocks appear to share the common feature of predicting that when the measuring process has been completed, differentiation (and, in at least some instances, cessation of division) follows necessarily. For example, if the period of division were limited by the accumulation of cellular damage or telomere shortening, then these events could not occur in cells stimulated to divide beyond their "normal" limit, for these events should by themselves be sufficient to limit cell division. Similarly, if the functioning of the biological clock relied simply on the buildup of a transcription factor to a level required to induce differentiation, then accumulation of such a factor should not occur in cells prevented from differentiating.

One experimental system that can be used to analyze the relationship among the measurement of elapsed time, the cessation of cell division, and the onset of differentiation is the timed differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells into oligodendrocytes. O-2A progenitors from embryonic rat CNS can be cultured in such a manner as to generate the first oligodendrocytes at a time equivalent to the day of birth, when the first oligodendrocytes appear *in vivo* (Abney *et al.*, 1981; Raff *et al.*, 1985, 1988). This appropriately scheduled differentiation in culture requires the presence of cortical astrocytes (Raff *et al.*, 1985) or purified platelet-derived growth factor (PDGF) (Raff *et al.*, 1988), an O-2A progenitor mitogen secreted by cortical astrocytes (Noble and Murray, 1984; Noble *et al.*, 1988; Richardson *et al.*, 1988). For example, in the presence of PDGF, optic nerve cultures from Embryonic Day 18 (E18) rats would generate the first oligodendrocytes after 3 days *in vitro*, while cultures from E19 rats would do so after 2 days (Raff *et al.*, 1988). That the scheduled differentiation of oligodendrocytes relied on the ability of O-2A progenitors to measure elapsed time was implied by the observation that clonally related O-2A progenitors generally ceased proliferating and differentiated within one division of each other, even if grown in separate tissue culture dishes after their first division (Temple and Raff, 1986). Synchronous differentiation of clonally related O-2A progenitors is also observed if cells are grown in chemically defined medium containing PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Thus, existing observations suggest that the appropriately timed generation of oligodendrocytes relies on a cell-intrinsic clock that resides within the O-2A progenitor and measures cell divisions or some other parameter (Temple and Raff, 1986).

In contrast to the behavior of O-2A progenitors stimulated to divide with PDGF, the appropriately timed generation of oligodendrocytes does not occur if cultures of optic nerve cells are grown in the absence of mitogen or are treated simultaneously with PDGF and basic fibroblast growth factor (bFGF). In the absence of mitogen, O-2A progenitors differentiate rapidly and prematurely into oligodendrocytes without dividing (Raff *et al.*, 1983; Noble and Murray, 1984; Temple and Raff, 1985). The diametrically opposite result is obtained if O-2A progenitors are grown in the presence of PDGF plus bFGF, a condition in which continuous division of O-2A progenitors is maintained in the absence of differentiation (Bögler *et al.*, 1990; Groves *et al.*, 1993). These latter results indicate that O-2A progenitors are not intrinsically limited to a relatively small number of divisions

before differentiating. Rather, such a limit represents a pattern of division and differentiation observed only when cells are grown specifically in the presence of PDGF.

The ability to promote continuous division of O-2A progenitors in the absence of differentiation by treatment with PDGF plus bFGF has offered us the opportunity to study in more detail the relationship between the measurement of time by a biological clock and the onset of differentiation and cessation of division. Our data show that prolonged exposure to PDGF plus bFGF alters the behavior O-2A progenitors subsequently exhibit in response to PDGF alone. It is also shown that a brief exposure to bFGF, in the continued presence of PDGF, did not alter the timing of differentiation of O-2A progenitors into oligodendrocytes. These results suggest that the measurement of time still occurs under conditions in which division continues indefinitely in the absence of differentiation. This implies that the measurement of elapsed time can be separated mechanistically from the mechanisms that control the onset of differentiation or the cessation of cell division.

MATERIALS AND METHODS

Analysis of Small Populations and Clones of Optic Nerve Cells

Primary optic nerve cultures and purified cortical astrocytes were established as described previously (McCarthy and De Vellis, 1980; Raff *et al.*, 1983; Noble and Murray, 1984; Raff *et al.*, 1985). For the period of culture in the presence of PDGF plus bFGF optic nerve cells were seeded in poly-L-lysine-coated 25-cm^2 flasks at 200,000 to 300,000 cells per flask in DMEM-BS, a chemically defined medium (a modification of the medium described by Bottenstein and Sato, 1979; Bögler *et al.*, 1990). Bulk optic-nerve-cell cultures were given 10 ng/ml of recombinant human PDGF A-chain homodimer [Chiron Corporation (a kind gift of Dr. C. George-Nascimento) or Promega] and recombinant human bFGF (Boehringer-Mannheim or Promega) each day and half the medium was changed every other day. Once a week the cells were passaged by trypsinization in calcium- and magnesium-free DMEM containing 200 μg/ml EDTA and 6000 U/ml trypsin, followed by trypsin inhibitor (added 1 part in 3.5; 0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml bovine pancreas DNase, and 3 mg/ml BSA fraction V in DMEM; Sigma). An aliquot of cells was removed for analysis at the times indicated under the Results section. The remainder of the cells were returned to bulk culture as above.

For analysis of small populations of optic nerve cells (Fig. 1), cocultures were established with cortical astrocytes, which produce PDGF (Noble *et al.*, 1988) and maximize optic nerve cell viability (Temple and Raff, 1986; Raff *et al.*, 1988; see also Barres *et al.*, 1992). As the age of the cortical astrocyte cultures could conceivably affect the behavior of O-2A progenitors (see Lillien and Raff, 1990, for one such phenomenon), fresh cultures of astrocytes were prepared according to identical schedules for each analysis. Astrocyte cultures were equivalent to P21 at the time that optic nerve cells were plated on them, an age when O-2A progenitor division and differentiation are still occurring *in vivo* (Miller *et al.*, 1985). Furthermore, these astrocyte cultures were made in the same way as those used previously to establish that astrocytes make PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Monolayers of 20,000 cortical astrocytes per poly-L-lysine-coated coverslip (Chance Propper No. 1, 13 mm) were cultured in 0.5 ml of DMEM containing 10% fetal calf serum (DMEM-FCS; Gibco BRL), which was changed 2 days later. After 3 days the cultures were irradiated with 2000 rads of X rays, washed once, and then fed with 0.5 ml DMEM-BS. One day later 100 μl of filter-sterilized DMEM-BS conditioned for 48 hr by cortical astrocytes (from a flask of the same cortex preparation that had not been passaged) was added to each well shortly before the optic nerve cells. Five hundred optic nerve-derived cells were delivered to the supernatant of these cultures in 10 μl. Approximately 50 to 100 cells were observed after 1 day on the coverslips covering part of the bottom of the well (Fig. 1). Half of the medium was replaced every other day, and growth factors were added daily.

For analysis of O-2A progenitor clones (Fig. 2) 1000 cortical astrocytes were plated in 10 μl of DMEM-FCS per Terasaki microwell and the plates incubated upside down for the first 48 hr, so that the astrocytes attached predominantly to the sides of the wells. This facilitated observation of the optic nerve cells subsequently plated into the microwells. These cultures were treated identically to those on coverslips described above, except: (a) instead of changing the medium after 2 days, 10 μl of DMEM-FCS was added; (b) they were not washed after irradiation before the medium was replaced with 10 μl of DMEM-BS; and (c) no conditioned medium was added separately, but the final dilutions of optic nerve cells were made in astrocyte-conditioned medium. A variety of dilutions of optic nerve-derived cells, designed to deliver between 1 and 10 cells per 10 μl per well, were plated into the Terasaki wells. The next day, and every 2 days after that, 10 μl of the medium was removed and the cultures were observed, and 10 μl DMEM-BS containing 10 μg/ml PDGF was replaced. After oligodendrocytic differentiation was judged to be complete on the basis of the morphology of visible cells, the cells

TABLE 1
bFGF ATTACHES TO THE MATRIX OF E19 OPTIC NERVE CULTURES

	E19 cells grown for first 24 hr in:	ECM incubated with:	Indicator cells cultured in:	% O-2A lineage cells GalC$^+$ after 4 days *in vitro*
1	—	—	PDGF	46.9 ± 3.2
2	—	—	PDGF + bFGF	3.3 ± 0.4
3	PDGF	PDGF	PDGF	57.4 ± 2.3
4	PDGF	PDGF	PDGF + bFGF	5.1 ± 0.8
5	bFGF	—	PDGF	4.0 ± 0.4
6	bFGF	—	PDGF + bFGF	3.2 ± 0.9
7	—	bFGF	PDGF	3.7 ± 0.4
8	—	bFGF	PDGF + bFGF	4.3 ± 0.6

Note. Exposure of E19 optic nerve cultures to bFGF either before or after lysis results in an extracellular matrix that inhibits the differentiation of O-2A progenitors in conjunction with PDGF. E19 optic nerve cultures were grown in the absence of growth factor (rows 1, 2, 7, and 8) in the presence of PDGF (rows 3 and 4) or in the presence of bFGF (rows 5 and 6) before they were lysed by hyposmotic shock. The ECM was then incubated for 24 hr in the absence of growth factor (rows 1, 2, 5, and 6) in PDGF (rows 3 and 4) or bFGF (rows 7 and 8) before indicator cells derived from 7-day-old optic nerve were plated onto the ECM and grown for a further 4 days in the presence of PDGF (odd-numbered rows) or PDGF plus bFGF (even-numbered rows). Data are presented as means ± SEM of three experiments.

were prepared for immunocytochemistry. The time required for oligodendrocytic differentiation to appear complete varied from culture to culture and depended on the size of the clones. The smallest clones (Fig. 2C) appeared to have differentiated after 3 days or less and were prepared for immunocytochemistry at that time. The largest clones were prepared for immunocytochemistry 10 days after optic nerve cells were plated into the microwells. Observations made before and after immunocytochemistry were compared, and only if they were sufficiently similar so that it appeared beyond reasonable doubt that a group of cells was derived from the single O-2A progenitor observed on Day 1 was the clone allowed into the data set shown in Fig. 2.

Analysis of Embryonic Optic Nerve Cultures

Initial experiments showed that E19 cultures that were given bFGF only on Day 1 behaved identically to those that received bFGF every day over a 10-day period: no oligodendrocytes appeared (data not shown; all cultures received PDGF every day). One possibility was that bFGF was persisting in these cultures due to interactions with the extracellular matrix (ECM; for review see Gospodarowicz *et al*, 1987; see also Lillien and Raff, 1990). To investigate this directly, the interaction of bFGF with the ECM of E19 optic nerve cultures was studied. E19 optic nerve cells were plated on coverslips and cultured in the absence of factors or in the presence of either PDGF or bFGF (10 ng/ml each) for 24 hr. These cultures were then subjected to hyposmotic lysis after 24 hr to generate an adherent cell lysate which consists predominantly of ECM (as in Lillien and Raff, 1990). Coverslips were then washed once with PBS and twice with DMEM-BS, incubated for 24 hr in DMEM-BS, or in DMEM-BS containing either PDGF or bFGF (20 ng/ml), and washed twice in DMEM-BS. Indicator cells from P7 optic nerve cultures maintained in PDGF plus bFGF for 2 days were then plated onto the ECM and grown for a further 4 days in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) before they were prepared for immunocytochemistry. If bFGF was present either before lysis, or after lysis but before the indicator cells were added, differentiation was inhibited as much as if bFGF was present after the indicator cells were added (Table 1, all indicator cell cultures received PDGF). In contrast, if the cultures saw either no growth factors before the indicator cells were plated, and then only PDGF, or if they saw only PDGF throughout, considerable differentiation occurred (Table 1). Therefore, it appears that bFGF became strongly attached to the ECM of these cultures and was able to affect the differentiation of O-2A progenitors in that attached form. In order to be sure to eliminate any bFGF attached to the ECM after the first 24 hr of culture, E19 optic nerve cultures were established in bulk (as above) and passaged by trypsinization after 24 hr. Cells were then plated on poly-L-lysine-coated coverslips at a density of 10,000 cells/coverslip in DMEM-BS. Cultures received either PDGF or PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF on a daily basis, at 10 ng/ml each. Approximately half the DMEM-BS was changed every 2 days, and cells were prepared for immunocytochemistry at the times indicated.

PDGF Receptor Analysis

For the PDGF receptor analysis shown in Figs. 4 and 5, optic nerve cultures were established in bulk and treated as described earlier. Aliquots of cells removed for analysis were plated directly onto poly-L-lysine-coated coverslips at a density of 3000 cells per coverslip. They were cultured for 48 hr in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) and prepared for immunocytochemistry.

Immunocytochemistry

The antibodies used were monoclonal anti-galactocerebroside (anti-GalC) antibody (Ranscht *et al*, 1982) and monoclonal antibody A2B5 (Eisenbarth *et al*, 1979).

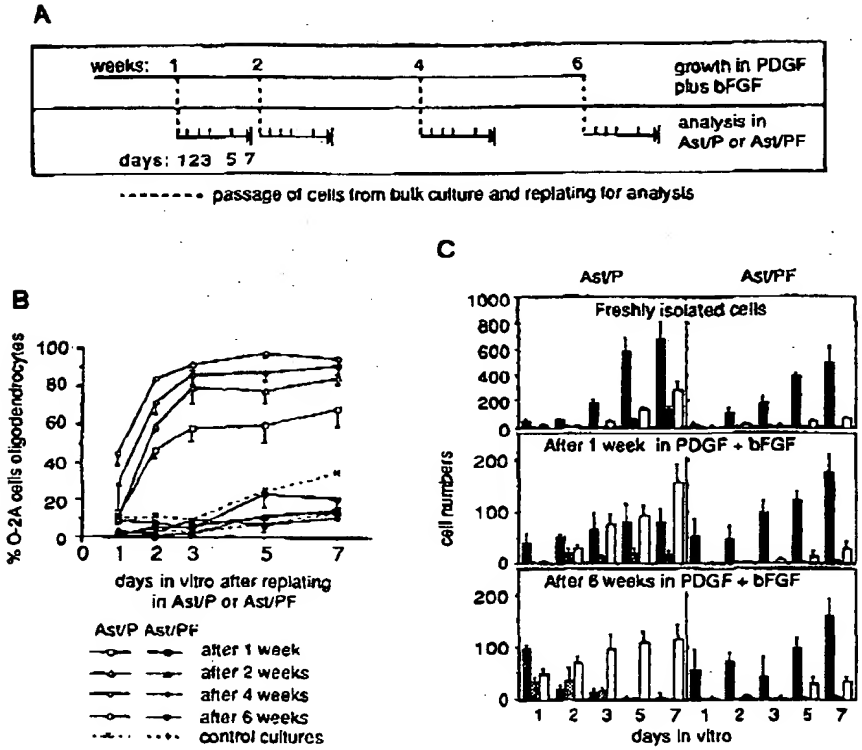

FIG. 1. Culture in PDGF plus bFGF leads to a gradual increase in the proportion of O-2A progenitors that no longer divide in response to Ast/P. (A) A schematic representation of the experimental design. Optic nerve cultures from 7-day-old rats were established in bulk for the period of culture in PDGF plus bFGF. Cells were passaged weekly, an aliquot was removed for analysis after 1, 2, 4, and 6 weeks, and the remaining cells were returned to bulk culture. Analysis was performed in coculture with cortical astrocytes and exogenous PDGF (Ast/P). (B) The percentage of O-2A lineage cells that were GalC$^+$ oligodendrocytes in optic nerve cell/astrocyte cocultures is shown graphed against time in coculture. Cocultures received either PDGF (Ast/P) or PDGF plus bFGF (Ast/PF). Cells were either derived from PDGF plus bFGF-treated bulk cultures (solid lines; mean ± SD of three or four coverslips per point) or were freshly isolated from optic nerve (dashed lines; control cells were analyzed in parallel with each experimental curve and the data pooled; mean ± SEM). For control cells only, the number of oligodendrocytes found in Ast/PF cultures on Day 1 was subtracted from all the control-cell data to allow a direct comparison with experimental cultures which contained almost no oligodendrocytes at the time of plating. As no new oligodendrocytes are generated in Ast/PF this number most closely resembles the number of oligodendrocytes initially plated. Only half of the error bars are shown for clarity, and if no error bars are shown then the SD or SEM was smaller than the radius of the plot symbol. Data is from one representative experimental series of three. (C) The numbers of A2B5$^-$GalC$^-$ O-2A progenitors (black bars), A2B5$^+$GalC$^+$ immature oligodendrocytes (stippled bars), or A2B5$^-$GalC$^+$ oligodendrocytes (white bars) in optic nerve cell/astrocyte cocultures are shown, graphed against time in coculture. Optic nerve cells were freshly isolated (top panels) or had been cultured in PDGF plus bFGF for 1 week (middle panels) or for 6 weeks (bottom panels). During the time shown parallel cultures received either PDGF only (Ast/P) or PDGF plus bFGF (Ast/PF). None of the cultures showed significant oligodendrocytic differentiation if cultured in PDGF plus bFGF: the number of oligodendrocytes shown in the right panels is similar to the numbers seen in astrocyte cultures to which no optic nerve cells were added. Data are means ± SD of three or four coverslips and are part of one representative experimental series of three.

The A2B5 monoclonal antibody specifically labels O-2A lineage cells in these cultures and is an IgM (Raff *et al.*, 1983), while anti-GalC, an IgG3, specifically labels oligodendrocytes (Raff *et al.*, 1978). Fluorescein- and rhodamine-conjugated second-layer antibodies against monoclonal antibodies were from Southern Biotechnology and were used at a dilution of 1:100. Fluorescein-conjugated anti-rabbit antibodies were from Tioga (USA) and were used at 1:200. Standard immunofluorescence to identify cells of the O-2A lineage was performed as described (Raff *et al.*, 1988; Noble and Murray, 1984; Bögler *et al.*, 1990). Anti-PDGF receptor immunofluorescence was done as follows. Cultures were rinsed in phosphate-buffered saline, fixed for 10 min in 4%

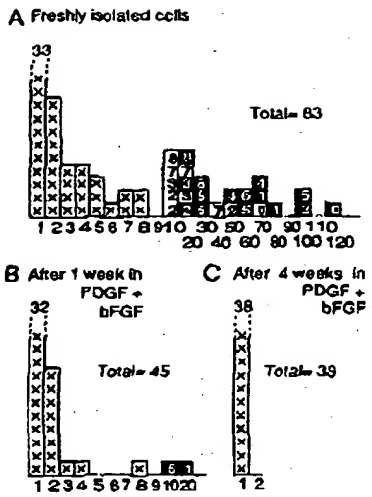

FIG. 2. Prolonged culture in PDGF plus bFGF leads to a reduction in the size of clones derived from individual O-2A progenitors. A stem and leaf plot (as in Temple and Raff, 1986) showing the reduction in clone size in the presence of astrocytes and PDGF, after switching from culture in PDGF plus bFGF. Each 'x' or number represents one clone. To obtain the number of cells in a given clone either read the number below the column in which the 'x' is or add the number in the graph (units) to the number under the column (tens). For example in the upper panel there are three clones of five cells and five clones that are between 10 and 20 cells consisting of 12, 12, 13, 17, and 18 cells. The total number of clones analyzed under each condition is indicated. The experimental results are from two separate bulk cultures that were analyzed after 1 and 4 weeks of culture in PDGF plus bFGF; data from freshly isolated P7 rat optic nerve cultures analyzed in parallel with each experiment were pooled. Clones shown in black writing on white ground consisted entirely of GalC$^+$ oligodendrocytes; clones shown as white writing on black ground contained some A2B5$^+$ GalC$^-$ O-2A progenitors, and these cells always comprised less than a third of the total cells, and typically about 10%.

paraformaldehyde, and then exposed sequentially to the following in Hepes-buffered Hanks' balanced salt solution (Imperial Laboratories) containing 5% newborn calf serum and 5% goat serum (both from GibcoBRL): 25 min A2B5 and anti-GalC, 25 min anti-IgG-biotin, 25 min streptavidin-coumarin (Molecular Probes, Oregon) and anti-IgM-fluorescein, 10 min 0.1% v/v Triton X-100, 45 min anti-PDGF receptor antibody (1:500; UBI), two 5-min washes, 30 min anti-rabbit fluorescein, two 5-min washes. Cultures were viewed on a Zeiss Axiphot microscope equipped with phase contrast and epi-uv illumination and selective filters for fluorescein, rhodamine, and coumarin.

Immunoprecipitation and Western Blot Analysis

Cultures of NIH 3T3 mouse fibroblasts, Rat-2 fibroblasts, and O-2A progenitors were harvested before becoming confluent by rinsing in PBS and scraping in 600 μl Ripa buffer (150 m*M* NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 m*M* Tris, pH 8.0) containing 0.5 m*M* PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 40 μg/ml bestatin, and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation, and the supernatant was immunoprecipitated by the addition of 20 μl of R7 anti-PDGF receptor antiserum (Eriksson *et al.*, 1992) and 60 μl of protein A–Sepharose CL4B (Pharmacia) and incubating on ice for 60 min. The immunoprecipitate was washed three times in 500 μl of Ripa buffer, and an equal volume of 2× sample buffer was added. The precipitates were boiled, separated on an acrylamide gel, and blotted to Immobilon P membrane (method described in Harlowe and Lane, 1988). The immunoblot was preblocked for 4 hr in blotto (5% nonfat dry milk in phosphate-buffered saline, 0.02% azide), exposed overnight to R7 antibody (1:400), washed extensively and exposed to ^{125}I-donkey anti-rabbit antibodies for 1 hr, followed by several washes, and exposed to X ray film.

RESULTS

O-2A progenitors dividing and differentiating in the presence of cortical astrocytes or PDGF exhibit three observable behaviors from which the functioning of their cell-intrinsic clock can be inferred. The first behavior is the ability of populations of O-2A progenitors to continue to generate new oligodendrocytes for a prolonged period of time, a process that is dependent upon ongoing O-2A progenitor division and differentiation (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). In contrast, if O-2A progenitors are no longer able to divide, oligodendrocyte numbers rapidly increase initially and then show no further change when all the O-2A progenitors have differentiated (assuming that the fairly consistent rate of differentiation of individual cells (e.g., Raff *et al.*, 1984) is itself not changed by the experimental manipulations). The second behavior that can be observed is the generation of clones of oligodendrocytes derived from a single O-2A progenitor. The size of the clone depends on the length of the limited period of division elicited by PDGF and so reflects the period of time that remained to be measured by the founding O-2A progenitor before differentiation was initiated (Temple and Raff, 1986). The third behavior is exhibited by populations of O-2A progenitors derived from embryonic optic nerve. Such populations give rise to the first oligodendrocytes *in vitro* at a time equivalent to the day of birth (Noble *et al.*, 1988; Raff *et al.*, 1988). This last behavior is the measure most likely to indicate whether the biological clock in O-2A progenitors is functioning appropriately.

The effect of preventing differentiation while stimulating division for varying periods of time has been experimentally determined for all three of these behaviors. The experimental design used for the analyses of the first two behaviors (experiments reported in Figs. 1 and 2) was to grow O-2A progenitors (derived from the optic nerves of 7-day-old rats) under conditions that prevented differentiation (PDGF plus bFGF) and to switch cells after different durations of culture to conditions that promoted timed differentiation (PDGF but not bFGF). The ability of these cells to divide and differentiate was then compared to that of freshly isolated control O-2A progenitors. Accordingly, optic nerve cells were established in bulk culture and given PDGF plus bFGF daily. Cultures were passaged weekly, and aliquots of cells were removed and replated in the presence of purified cortical astrocytes for analysis (see Fig. 1A). Coculture with cortical astrocytes was used as astrocytes make PDGF and promote timed differentiation of O-2A progenitors (Noble *et al.*, 1988; Richardson *et al.*, 1988) as well as maximizing O-2A lineage cell viability (Temple and Raff, 1986; Barres *et al.*, 1992), thereby allowing the analysis of small populations of cells or even individual clones. Astrocyte-optic nerve cocultures received either no additional factors, PDGF, or PDGF plus bFGF (referred to as Ast, Ast/P, or Ast/PF, respectively) and were analyzed by immunocytochemistry after 1, 2, 3, 5, and 7 days. As expected from the fact that astrocytes make PDGF, we observed no differences between cultures that received no additional factors (data not shown) and those that received PDGF alone and present data only on cells that were switched to Ast/P.

Populations of freshly isolated O-2A progenitors cultured in the presence of PDGF exhibit both O-2A progenitor self-renewal by division and oligodendrocyte generation by differentiation (Noble and Murray, 1984; Noble *et al.*, 1988; see also Wren *et al.*, 1992). In PDGF-treated cultures of optic nerve cells, the extent of O-2A progenitor self-renewal is related to the proportion of O-2A progenitors with some time remaining in their limited period of division, and so have not undergone timed differentiation. The capacity of O-2A progenitor self renewal to continue for prolonged periods in populations of cells is thought to reflect a heterogeneity in the duration of the mitotic period remaining in different clones of the starting population (Temple and Raff, 1986). As a consequence one cannot measure the timed differentiation of O-2A progenitors into oligodendrocytes in postnatal optic nerve cultures as a single endpoint. The changing numbers of O-2A progenitors and oligodendrocytes in a population analyzed at various time points can be used, however, to give some indication of the amount of time that remains to be measured by the

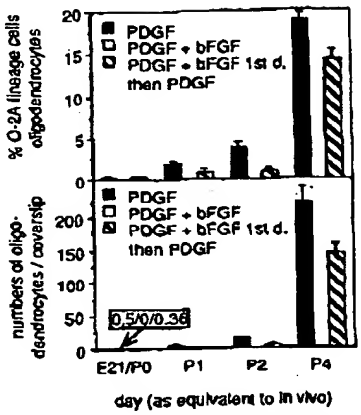

FIG. 3. Transient exposure to PDGF plus bFGF does not perturb the appearance of the first oligodendrocytes in embryonic optic nerve cultures. Data shows the percentage of O-2A lineage cells that were GalC⁺ oligodendrocytes (top) or the average number of oligodendrocytes seen per coverslip (bottom) in cultures derived from E19 optic nerve. The average number of oligodendrocytes per coverslip at the time equivalent to E21/P0 is shown as numbers (PDGF:0.5/PDGF plus bFGF:0/PDGF plus bFGF for first day, then PDGF: 0.36), as the bars are too small to be seen. Cultures were established in bulk for the first 24 hr and then passaged and replated on coverslips. They received either PDGF throughout, PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF only. Differentiation state was analyzed by immunocytochemistry at 2, 3, 4, and 6 days after dissection, shown as days equivalent to *in vivo*. Data are presented as means ± SEM of three experiments.

O-2A progenitors in that population. If, on average, a large amount of time remains in the limited period of division that precedes timed differentiation, then one would observe the continuous generation of both O-2A progenitors and oligodendrocytes. In contrast, if all the O-2A progenitors have completed the measurement of time and reached the end of their period of division, they would be expected to differentiate rapidly into oligodendrocytes and to show little sign of self-renewal.

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Gradual Reduction in the Duration for Which They are Subsequently Mitotically Responsive to PDGF Alone

Analysis of populations of O-2A progenitors that had been switched from culture in PDGF plus bFGF to Ast/P showed a dramatic increase in the proportion of O-2A lineage cells that were oligodendrocytes after the first 3 days in Ast/P, when compared to freshly isolated O-2A progenitors (Fig. 1B). Furthermore, the proportion of the starting population that differentiated into oligodendrocytes during the first 3 days of culture in Ast/P

TABLE 2
O-2A PROGENITORS THAT ARE NO LONGER MITOTICALLY RESPONSIVE TO PDGF EXPRESS PDGF RECEPTORS

Time of culture in PDGF plus bFGF	Percentage of O-2A progenitors that were PDGF receptor⁻	
	PDGF	PDGF + bFGF
Control cells	96.1 ± 1.1	95.9 ± 1.1
1 week	96.0 ± 1.4	96.8 ± 0.9
2 weeks	98.1 ± 0.5	98.0 ± 0.7
4 weeks	99.6 ± 0.3	98.7 ± 0.5
6 weeks	97.9 ± 0.8	98.3 ± 0.5

Note. The percentage of O-2A progenitors that were PDGF receptor⁻ after various times of culture in the presence of PDGF plus bFGF followed by a further 2 days of culture in PDGF or PDGF plus bFGF. For culture in the presence of PDGF plus bFGF cells were treated as described for the experiments shown in Figs. 1 and 2. Aliquots of cells were removed after 1, 2, 4, and 6 weeks and replated at a density of 3000 cells per coverslip. They were treated with either PDGF or PDGF plus bFGF and prepared for immunocytochemistry after 2 days. They were labeled with anti-PDGF receptor antibodies, as well as A2B5 and anti-GalC antibodies. The proportion of A2B5⁺ GalC⁻ O-2A progenitors that were also labeled by the anti-PDGF receptor antibodies is shown. Data is mean ± SEM for between 6 and 10 coverslips from two separate experiments.

correlated well with the duration of prior culture in PDGF plus bFGF (Fig. 1B): the longer cells had been grown in the presence of PDGF plus bFGF the greater the proportion of O-2A lineage cells that differentiated into oligodendrocytes after 3 days in Ast/P. Three days after switching cells to Ast/P the rate of increase of the percentage of oligodendrocytes slowed, either because the rate of O-2A progenitor self-renewal became similar to the rate of oligodendrocyte generation (after 1, 2, and 4 weeks in PDGF plus bFGF) or because there were almost no O-2A progenitors left at this time (after 6 weeks in PDGF plus bFGF). As expected, all cultures in Ast/PF showed little increase in the proportion of O-2A lineage cells that were oligodendrocytes as under these conditions O-2A progenitor division occurred in the absence of oligodendrocyte differentiation (Fig. 1B).

Examination of the data shown in Fig. 1B in terms of cell numbers suggested that after 6 weeks in PDGF plus bFGF, no significant amount of O-2A progenitor division occurred in Ast/P (Fig. 1C, bottom left panel shows no change in the sum of the bar heights; the culture conditions prevent any significant cell death: see Barres *et al.*, 1992). In contrast, freshly isolated populations of O-2A progenitors, or cells switched after 1 week of culture in PDGF plus bFGF, showed a combination of O-2A progenitor self-renewal and oligodendrocyte generation in Ast/P (Fig. 1C, left panels). The behavior of O-2A progenitors switched to Ast/P after only 1 week of culture in PDGF plus bFGF was intermediate between that of the cells grown for 6 weeks and freshly isolated cells: although some O-2A progenitor division occurred (as indicated by an increase in cell number), a larger proportion of cells in the 1 week pre-treated culture became oligodendrocytes within 3 days than in cultures of freshly isolated cells (compare Fig. 1C left panels). As expected, all Ast/PF cultures showed O-2A progenitor division and no significant oligodendrocyte generation over the time points examined (Fig. 1C).

Cells differentiating from an A2B5⁺GalC⁻ O-2A progenitor into an A2B5⁻GalC⁺ oligodendrocyte are transiently A2B5⁺GalC⁺, and the proportion of O-2A lineage cells that occupy this compartment at any given time is related to the rate of differentiation of the population as a whole. Examination of this A2B5⁺GalC⁺ compartment also shows that pretreatment with PDGF plus bFGF is associated with an increase in the number of differentiating cells seen at early time points (Fig. 1C, stippled bars). Again, 6 weeks of prior culture in PDGF plus bFGF had a more marked effect than 1 week of prior culture (Fig. 1C, left panels). After 6 weeks in the presence of PDGF plus bFGF, very few A2B5⁺GalC⁺ cells were found in these culture after the 3-day time point (Fig. 1C). In contrast, A2B5⁺GalC⁺ cells were found on all days examined in cultures grown in the presence of PDGF plus bFGF for 1 week before switching to Ast/P. It is interesting to note, however, that significant numbers of such cells were seen in these cultures several days before their presence in cultures derived from freshly isolated cells. Thus, these data also indicate that the yield of oligodendrocytes within a given time period of growth in Ast/P is increased by prior culture in PDGF plus bFGF and further indicate that the rate of differentiation itself is probably not altered from the figure of 3 days reported in earlier studies (Raff *et al.*, 1984).

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Stepwise Reduction in the Size of the Oligodendrocyte Clones Subsequently Generated in Response to PDGF Alone

Clonal analysis allows direct measurement of the number of cell divisions that the founding cell of a clone and its progeny underwent. In the case of O-2A progenitors dividing in response to PDGF, clone size depends on the length of the limited mitotic period that precedes timed differentiation, assuming that no significant amount of cell death occurred (Temple and Raff, 1986). Therefore, clones derived from O-2A progenitors that

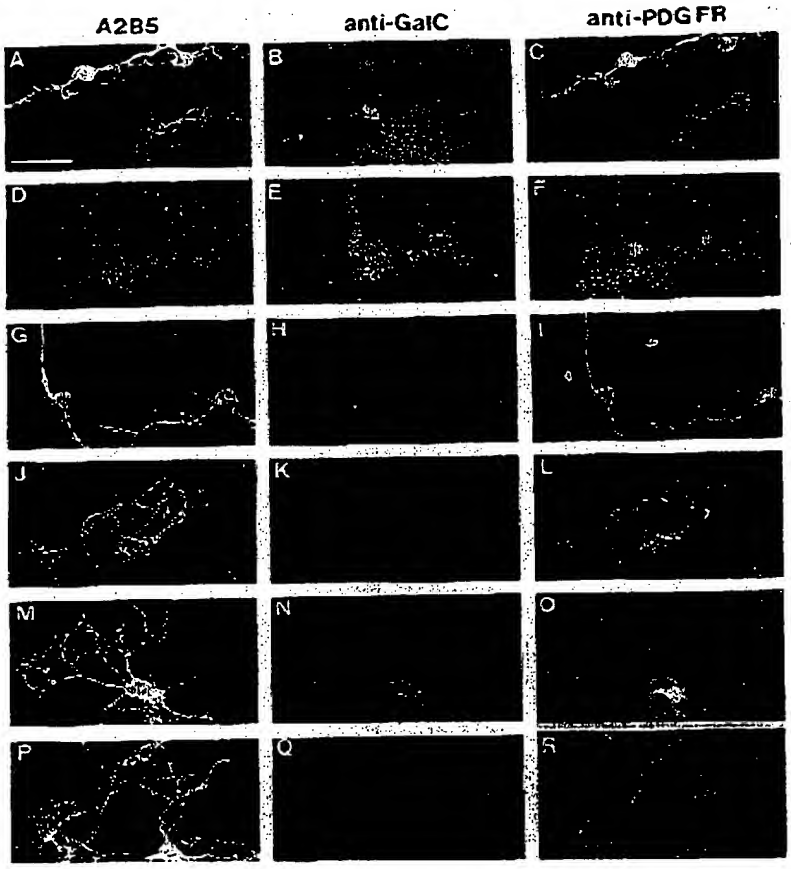

FIG. 4. O-2A progenitors cultured in PDGF plus bFGF for 6 weeks followed by 2 days in PDGF continue to express PDGF receptors. Three-color immunocytochemistry of optic nerve cultures using monoclonal A2B5 (via rhodamine) and anti-GalC (via coumarin) and polyclonal anti-PDGF receptor antibodies (via fluorescein). Cells were either derived directly from P7 optic nerve (A to I) or had undergone 6 weeks of preculture in PDGF plus bFGF (J to R). They were cultured for 2 days in the presence of PDGF alone (A to F and J to O) or in the presence of PDGF plus bFGF (G to I and P to R). $A2B5^-GalC^-$ O-2A progenitors were PDGF receptor$^+$ under all conditions (A to C; G to L; P to R). In contrast, $A2B5^-GalC^+$ oligodendrocytes such as the one indicated by the arrow in B, were negative for the PDGF receptor. D to F and M to O show $A2B5^+GalC^+$ immature oligodendrocytes that are PDGF receptor$^+$. In the control cultures that were derived from P7, optic nerve cells that were PDGF receptor$^+$, but did not label with either A2B5 or anti-GalC, were common (short arrows in I). These cells were probably pial meningeal cells. The scale bar in A is 30 μm.

had been grown in PDGF plus bFGF for varying periods of time, or had been freshly isolated from optic nerve, were analyzed in insulin containing coculture with cortical astrocytes (Noble *et al.*, 1988; Raff *et al.*, 1988; Barres *et al.*, 1992), following the method of Temple and Raff (1986). Cultures were observed the day after optic nerve cells were placed in microculture and every other day after that. When oligodendrocytic differentiation was judged to be complete by cell morphology (after between 3 and 10 days, depending on how many rounds of division occurred) cultures were prepared for immunocytochemistry to confirm the differentiation state of the cells and to facilitate counting of the cells.

Clones derived from freshly isolated O-2A progenitors showed a wide range of sizes as expected (Fig. 2A; Temple and Raff, 1986). In contrast, after 4 weeks in PDGF plus bFGF all of the 38 O-2A progenitors analyzed generated clones of 1 oligodendrocyte (Fig. 2C), suggesting that these O-2A progenitors were no longer induced to divide by PDGF when grown in single-cell microculture. None of the founding cells was seen to divide and all differentiated within 3 days of plating

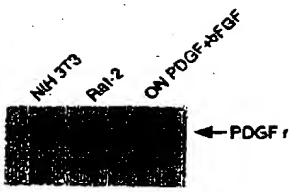

FIG. 5. O-2A progenitors cultured for 7 weeks in PDGF plus bFGF express PDGF-α receptor. Anti-PDGF-α receptor Western blot of anti-PDGF-α receptor immunoprecipitates of Rat-2, NIH 3T3 fibroblasts, and an optic nerve culture that had been grown for 7 weeks in the presence of PDGF plus bFGF. The position of the PDGF-α receptor is indicated, and these bands migrated at approximately 180 kDa.

into the microwells. O-2A progenitors that had been first grown for a week in PDGF plus bFGF showed behavior that was intermediate between that of freshly isolated cells and cells that had been in the presence of PDGF plus bFGF for 4 weeks (Fig. 2B). The largest clone derived from freshly isolated cells consisted of 120 cells, implying that the founder O-2A progenitor of this clone underwent at least seven divisions. After 1 week in PDGF plus bFGF clones of 21 cells or fewer were obtained, suggesting that O-2A progenitors underwent a maximum of five divisions after switching to Ast/P. It should be noted that some of the clones shown in Fig. 2 contained A2B5$^+$GalC$^-$ O-2A progenitors at the time that the cultures were fixed (white letters on black background). These cells had appeared to be oligodendrocytes when they were observed live under the phase-contrast microscope as they had a more complex, branched morphology than typical bipolar O-2A progenitors. As in the course of oligodendrocytic differentiation, morphology changes before antigenic phenotype (Noble and Murray, 1984; Raff *et al.*, 1985; Temple and Raff, 1986) and is generally associated with a cessation of division (Small *et al.*, 1987; Noble *et al.*, 1988), it is most likely that these cells were at an early stage of differentiation. The presence of a few A2B5$^+$GalC$^-$ multipolar O-2A progenitors in some clones is consistent with previous observations (Temple and Raff, 1986).

A Short Exposure to bFGF Does Not Alter the Timing of the Appearance of the First Oligodendrocytes in PDGF-Treated Embryonic Optic Nerve Cultures

Analysis of the behavior of small populations (Fig. 1) and clones (Fig. 2) suggested that culture in PDGF plus bFGF led to a gradual shortening of the period of time during which O-2A progenitors would subsequently divide in response to Ast/P. One possible explanation is that cells registered the passing of time in the presence of PDGF plus bFGF and that exposure to bFGF in the presence of PDGF did not prevent the measurement of time in cultures of postnatal optic nerve. An alternative explanation would be that any exposure to bFGF dramatically increases the yield of oligodendrocytes produced within the first few days of subsequent growth in Ast/P. To test this possibility we used the sensitive assay for the correct functioning of the clock provided by the timed appearance of oligodendrocytes in cultures of embryonic optic nerve. The first oligodendrocytes appear in embryonic optic nerve cultures treated with PDGF at a time equivalent to birth, and invariably do so within a 24-hr window (Raff *et al.*, 1988). Therefore, we exposed embryonic optic nerve cultures transiently to bFGF (in the continued presence of PDGF) and followed the appearance of oligodendrocytes.

Transient exposure to bFGF in the continued presence of PDGF did not alter the time of appearance of the first oligodendrocytes in embryonic optic nerve cultures from that observed in control cultures that received PDGF throughout the experiment (Fig. 3). However, slightly fewer oligodendrocytes appeared over the course of the experiment in cultures that transiently received bFGF, both in terms of cell numbers and proportion of O-2A lineage cells. This raises the possibility that short-term exposure to bFGF increased the number of divisions that some clones of cells underwent, as has been suggested by others (McKinnon *et al.*, 1990). Cells that received either PDGF or PDGF plus bFGF throughout behaved as expected from previous studies: in PDGF alone the first oligodendrocytes were generated after 2 days (Fig. 3; Raff *et al.*, 1988), while in PDGF plus bFGF no oligodendrocytes were generated (Fig. 3; Bögler *et al.*, 1990).

O-2A Progenitors That Are Mitotically Unresponsive to PDGF Continue to Express PDGF-α Receptors

One possible explanation for the observation that prolonged culture in the presence of PDGF plus bFGF led to an inability of some or all O-2A progenitors to subsequently respond to Ast/P is that the levels of PDGF-α receptor declined. In order to investigate this possibility, we asked whether long-term cultures of O-2A progenitors grown in the presence of PDGF and bFGF continued to express PDGF-α receptors. We also tested whether O-2A progenitors grown for various periods of time in PDGF plus bFGF and then switched to the presence of PDGF alone for 2 days expressed PDGF-α receptors. Bulk optic nerve cultures derived from P7 optic nerve were established as for previous experiments. Cells were removed from PDGF plus bFGF after 1, 2, 4, and 6 weeks and analyzed by PDGF receptor immunocytochemistry after a further 2 days, either in

the presence of PDGF plus bFGF or in PDGF alone (Table 2 and Fig. 4). This time point was chosen as it represents a compromise between allowing the maximum time for the levels of receptor to decline if it were no longer being synthesized and retaining enough A2B5$^+$GalC$^-$ O-2A progenitors present in all the cultures to be able to analyze a reasonably sized population of cells.

Greater than 95% of A2B5$^+$GalC$^-$ O-2A progenitors showed a clear reaction with anti-PDGF receptor antibodies in all cultures examined, suggesting that they expressed PDGF-α receptor (Table 2 and Fig. 4) despite being refractory to the mitogenic effects of PDGF. In contrast, A2B5$^-$ GalC$^+$ oligodendrocytes were not labeled by the anti-PDGF receptor antibodies in any cultures (Fig. 4A to 4C). In both the control and experimental populations that received only PDGF, A2B5$^+$ GalC$^+$ immature oligodendrocytes that were also PDGF receptor$^+$ were seen, suggesting that at least in some cases PDGF-α receptor levels did not decline until after differentiation was already in progress.

The cells that remained from the bulk culture of one of the above experiments after 6 weeks were returned to culture in the presence of PDGF plus bFGF for a further 7 days before being harvested for analysis by immunoprecipitation and Western blotting with an antiserum specific for the PDGF-α receptor (Eriksson *et al*, 1992). As can be seen in Fig. 5 this optic nerve culture, which contained almost only O-2A progenitors, expressed levels of PDGF-α receptor similar to NIH 3T3 or Rat-2 fibroblasts.

DISCUSSION

When grown in the presence of PDGF, O-2A progenitors derived from optic nerves of perinatal rats undergo a limited period of division, at the end of which they switch from division to differentiation. The duration of this period of division is thought to be regulated by a cell-intrinsic clock. We examined whether O-2A progenitor behavior changed with elapsed time when cells were grown for varying periods of time under conditions that stimulate division and prevent differentiation. It was found that O-2A progenitors grown in the presence of PDGF plus bFGF, which together prevent differentiation, gradually became refractory to the mitogenic effect of subsequent culture in Ast/P in the absence of bFGF. The longer the period in PDGF plus bFGF, the greater the proportion of O-2A progenitors that differentiated without dividing after having been switched to Ast/P. Eventually, after 6 weeks in PDGF plus bFGF, almost all the O-2A progenitors differentiated without dividing in Ast/P. In addition, O-2A progenitors precultured in PDGF plus bFGF gave rise to smaller clones of oligodendrocytes, after switching to single cell culture in Ast/P, than did freshly isolated cells grown in parallel single cell microculture. After 1 week in PDGF plus bFGF the maximum clone size observed after switching to Ast/P was 21 cells compared to 120 cells seen in the largest clone derived from freshly isolated O-2A progenitors. Exposure to both mitogens for 4 weeks was sufficient to produce a population of O-2A progenitors that no longer divided after switching to Ast/P in single cell microculture. In contrast, a short exposure to PDGF plus bFGF did not result in any similar shortening of the period of division of O-2A progenitors after switching to PDGF alone.

We suggest that the simplest explanation of our results is that O-2A progenitors cultured in the presence of PDGF plus bFGF measure time and retain a memory of the fact that they have exceeded the number of divisions that they would normally undergo in the presence of PDGF alone. While it appears that this continued functioning of the biological clock was able to bring cells to the brink of differentiation, it was not sufficient to cause a cessation of cell division or to induce differentiation. These results represent an extension of previous studies of this phenomenon, in that the measurement of time by O-2A progenitors had previously only been observed as the timed switching of cells from a program of self-renewal by division to a program of differentiation, in the presence of PDGF (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al*, 1988; Raff *et al*, 1988). From these studies it was not possible to determine whether the clock is part of the mechanisms that regulate cell division and differentiation or is separate from them. The demonstration that the measurement of time apparently occurs under conditions where division continues in the absence of differentiation allows us to examine this issue.

Predictions about the behavior of the clock under conditions in which cells divide but do not differentiate differ depending on whether it is a separate and independent mechanism. If the clock were an integral part of either the cell division or differentiation machinery, it would follow that the clock would be inoperative under the growth conditions we examined. In detail, if the clock worked by limiting the number of cell divisions, for example by the loss of an activity required for execution of the cell cycle, then conditions under which this limit is removed would prevent the measurement of time. Similarly, if the clock operated by inducing differentiation after a set period of time, conditions which prevent differentiation would destroy the cell's ability to measure time. Our observations appear to rule out both of these groups of potential mechanisms, as our

data suggest that the measurement of time is ongoing under conditions that induce continued proliferation in the absence of differentiation. Instead, we would suggest that our results are more consistent with the hypothesis that the measurement of time is performed by a separate cellular mechanism that can interact with the mechanisms controlling division and/or differentiation, yet is distinct from these mechanisms.

It should be noted that our experiments do not address the questions of whether cell division is required for the functioning of the clock or whether it is the number of cell divisions that are being counted. In order to examine these issues it would be necessary to analyze the ability of cells to measure elapsed time in the absence of cell division. Therefore, while our hypothesis states that the clock is separate from the cell division machinery (i.e., does not function by imposing an irrevocable limit on the number of cell divisions), it does not state that the clock does not require cell division to function.

Previous analysis of O-2A progenitors in single cell culture has led to the estimate that a maximum of eight rounds of cell division occur in response to stimulation by purified astrocytes (Temple and Raff, 1986), which appear to exert their mitogenic effects through PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988; Richardson *et al.*, 1988). This estimate is at odds with our finding that growth for between 4 and 6 weeks in PDGF plus bFGF is required for O-2A progenitors to reach the point at which they are unresponsive to Ast/P (Figs. 1 and 2). O-2A progenitors grown in PDGF, or in PDGF plus bFGF, appear to have similar cell cycle times of approximately 20 hr (Noble *et al.*, 1988; Bögler *et al.*, 1990). At such a rate of division, at least 28 divisions (4 weeks; see Fig. 2) would be required to exhaust the measurement of time in the presence of PDGF plus bFGF.

One explanation for this difference between our observations and those of Temple and Raff (1986) would be that exposure to bFGF alters the clock by extending the period of time to be measured. This possibility has been raised in the context of a model for the clock whereby the measurement of time is ascribed to a reduction in the level of PDGF-α receptors, in a study where it was observed that bFGF could up-regulate the amount of PDGF-α receptor expressed by O-2A progenitors (McKinnon *et al.*, 1990). This possibility would not be inconsistent with our observation that a short exposure of embryonic optic nerve-derived O-2A progenitors to bFGF was associated with a slower production of oligodendrocytes, although the time of their first appearance was not changed (Fig. 3). However, other data from our study show that exposure to bFGF (in the presence of PDGF) for 1 week or longer leads to a reduction in the subsequent period of division in response to PDGF, suggesting that exposure to bFGF does not inevitably lead to an extension in the clock.

Another possible explanation for the discrepancy between our results and those of Temple and Raff (1986) is that the experimental conditions under which the measurement of time is examined can affect the result. When we analyzed individual O-2A progenitors as opposed to small populations, we observed that a shorter period of previous culture in PDGF plus bFGF resulted in the complete absence of division: for small population analysis 6 weeks was required, while for single cell analysis only 4 weeks sufficed (compare Figs. 1 and 2). This would suggest that any measure of the length of the PDGF responsive period derived from observations in single cell microculture may lead to an underestimate when compared to experiments using small populations. It is not clear whether these differences are due to interactions between O-2A progenitors, changes in the ratio of astrocytes to optic nerve cells, or other factors.

O-2A progenitors that differentiate under the control of the clock lose the ability to divide in response to PDGF before any overt phenotypic changes occur (Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). Oligodendrocytes are however not truly postmitotic as they retain the ability to divide in response to bFGF for a period of time in culture (Eccleston and Silberberg, 1985; Saneto and De Vellis, 1985; Noble *et al.*, 1988; Bögler *et al.*, 1990; Mayer *et al.*, 1993). It has, therefore, appeared probable that a key event in triggering timed differentiation in O-2A progenitors is the selective loss of mitotic responsiveness to PDGF. This has seemed unlikely to be due to a loss of PDGF receptors, as at least 50% of newly formed oligodendrocytes express detectable levels of PDGF receptors (Hart *et al.*, 1989b). Furthermore, the PDGF receptors on immature oligodendrocytes are able to transmit signals: PDGF is capable of inducing a rise in intracellular calcium (Hart *et al.*, 1989a) as well as the expression of the proto-oncogenes c-fos and c-jun (Hart *et al.*, 1992). In addition, PDGF can transiently act as a survival factor for oligodendrocytes (Barres *et al.*, 1992) and elevate protein levels of the transcription factor SCIP/Tst-1/Oct-6 in immature oligodendrocytes (O. Bögler, A. Entwistle, R. Kuhn, G. Lemke, and M. Noble, unpublished observation). Our observation that O-2A progenitors that have become refractory to the mitogenic effect of PDGF after prolonged culture in PDGF plus bFGF, but continue to express easily detectable levels of PDGF receptor, is therefore in agreement with the work of others. The possibility that changes too subtle to be discerned with

either anti-PDGF receptor antibodies (this study) or radiolabeled PDGF (Hart *et al.*, 1989b) underlie timed differentiation cannot, however, be excluded at present.

Recent studies conducted on fibroblast populations suggest that the type of phenomenon we have observed is not restricted to O-2A progenitor cells. These experiments have been performed on fibroblast populations derived from H-2K^b-tsA58 transgenic mice (Jat *et al.*, 1991), which can be induced to express the immortalizing tsA58 mutant of SV40 large T antigen immediately after dissection by growth of cells in the presence of interferon at 33°C. Thus, these cells can be exposed to a stimulus that allows them to continue dividing beyond their normal limited mitotic life span from the beginning of their *in vitro* growth, in analogy with the exposure of O-2A progenitors to PDGF plus bFGF. Fibroblasts derived from H-2K^b-tsA58 transgenic mice are only conditionally immortal, in that switch to growth in interferon-free medium at 39.5°C turns off function of the large T antigen, again in analogy with the switch of O-2A progenitors from growth in PDGF plus bFGF to growth in Ast/P. It was shown previously that inactivation of a conditional immortalizing stimulus in long-term rodent fibroblast cultures was associated with the cessation of growth and an accumulation of cells in the G1 and G2/M phases of the cell cycle, suggesting that the removal of active large T antigen led to the recapitulation of senescence (Grove and Cristofalo, 1977; Jat and Sharp, 1989). The more recent studies on fibroblasts derived from H-2K^b-tsA58 transgenic mice have indicated that in these cells the normal mitotic life span is measured despite the presence of large T antigen. Thus, cells switched to nonpermissive conditions after various numbers of passages under immortalizing conditions have a shorter remaining mitotic life span than control cells, with the extent of remaining mitotic life span being related directly to the length of time the cells were first grown under immortalizing conditions. Moreover, cells switched after the normal mitotic life span has elapsed rapidly cease cell division and become senescent (Ikram *et al.*, in press). These results suggest that cells prevented from undergoing senescence by the presence of an immortalizing oncogene retain cellular memory of having passed the number of cell divisions after which they would have ordinarily senesced, implying that the mechanism that regulates the onset of senescence measures time in the presence of an oncogene. The timing mechanisms that regulate senescence and oligodendrocyte differentiation therefore appear to be at least superficially similar in that they remain active when not being able to regulate the onset of the processes they normally control. The extent to which such processes are indeed controlled by similar mechanisms will only be established when the molecular basis of timed differentiation is understood.

We suggest that the data we have presented establish that the biological clock of O-2A progenitor cells is an autonomous mechanism distinct from the mechanisms which allow cell division or promote cell-type-specific differentiation. An elucidation of the molecular nature of the clock may be facilitated by the ability to generate cells which are no longer able to measure time, for example by prolonged culture of O-2A progenitors in PDGF plus bFGF or rodent fibroblasts harboring a temperature-sensitive SV40 large T antigen. Such cells could be used as targets for attempts to reconstitute the measurement of time and so may lead to the identification of the molecules involved.

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Table 3 Anti-PDGF antibodies block response of O-2A progenitors to type-1 astrocytes and PDGF, but not to FGF

	Radiolabelled O-2A progenitors (%)	
	No anti-PDGF	Plus anti-PDGF
ACM	63+4.2%	<1%
PDGF, 5 ng/ml	69+3.7%	<1%
FGF, 5 ng/ml	40+4.2%	44.5+2.5%

Cells were grown as in Table 1. Cultures received either the indicated mitogen, or mitogen plus 50 μg of rabbit anti-PDGF antiserum (purified Ig fraction, a kind gift of C. Heldin). When antibody was added, the ACM or non-conditioned medium with mitogen was preincubated with antibody for at least 1 h before addition to the cells. Cells were labelled with [^{3}H]-thymidine, immunolabelled, processed for autoradiography and scored as in Table 2.

the medium contained PDGF (data not shown). Thus, PDGF neither inhibited nor induced differentiation of O-2A progenitor cells into type-2 astrocytes, nor did it preclude differentiation of O-2A progenitor cells into oligodendrocytes. In these respects also, the effects of PDGF were identical to the effects of type-1 astrocytes.

To determine whether the effects of type-1 astrocytes were mediated by PDGF, we treated ACM, or medium containing PDGF or fibroblast growth factor (FGF) with affinity-purified anti-PDGF antibody. (As will be discussed elsewhere, FGF is also a mitogen for O-2A lineage cells, but does not mimic the effects of type-1 astrocytes on motility and differentiation.) These antibodies blocked the effects of ACM and PDGF, but did not block FGF-induced DNA synthesis in O-2A progenitors (Table 3), indicating that blocking was not due to toxic effects of the antibody.

Type-1 astrocytes also support the appropriately timed differentiation of embryonic O-2A progenitors grown *in vitro*[6]. As discussed in the accompanying paper[7], astrocyte-derived PDGF seems to play a key role in this effect and is by itself sufficient to promote the synchronous differentiation of clonally related and dividing progenitor cell families. Thus, in these respects also, PDGF completely mimics the effects of type-1 astrocytes.

The ability of a single molecule to replace type-1 astrocytes in modulating O-2A progenitor development *in vitro* suggests that these cells have a complex and constitutive behavioural phenotype, controlled by processes internal to the progenitors themselves. Progenitors stimulated to divide by an appropriate mitogen appear to be intrinsically migratory cells, with a bipolar morphology, which cease migration upon differentiating into multipolar oligodendrocytes; this differentiation seems to be controlled, at least in part, by internal clocks which may function by counting cell divisions. This programme does not, however, include astrocyte differentiation, which requires a separate inducing factor[10].

The observations that PDGF induced DNA synthesis at picomolar concentrations, the identical effects of PDGF and type-1 astrocytes on the division, differentiation and motility of O-2A progenitors and the ability of anti-PDGF antibodies to block the activity of astrocyte-conditioned medium all indicate that the astrocyte activity is a PDGF-like substance. In addition, messenger RNA for PDGF has been identified in purified astrocytes and these cells have been found to secrete PDGF *in vitro*[17] (W. Richardson, N. Pringle, M. Moseley, B. Westermark, & M. Dubois-Dalcq, manuscript submitted). Together, our studies indicate that PDGF may play an important role in gliogenesis in the CNS.

The ability of PDGF to promote division and migration of O-2A progenitor cells may be of particular interest in light of observations that PDGF can act as a chemotactic agent[11]. During embryogenesis, O-2A progenitors appear to populate the optic nerve by migrating from a germinal zone in or near the optic chiasm along the nerve towards the eye[5]. This directional migration could be due to movement along a gradient of a chemotactic substance, such as PDGF. In the adult animal, the ability of cells in a lesion site to secrete compounds which promote O-2A progenitor migration and division could be of value in the repair of demyelinating damage in the CNS. The controlled application of PDGF, or other chemotactic mitogens, might enhance these repair processes.

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Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture

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The various cell types in a multicellular animal differentiate on a predictable schedule but the mechanisms responsible for timing cell differentiation are largely unknown. We have studied a population of bipotential glial (O-2A) progenitor cells in the developing rat optic nerve[1] that gives rise to oligodendrocytes beginning at birth and to type-2 astrocytes[2] beginning in the second postnatal week[3]. Whereas, *in vivo*, these O-2A progenitor cells proliferate and give rise to postimitotic oligodendrocytes over several weeks[4,5], in serum-free (or low-serum) culture they stop dividing prematurely and differentiate into oligodendrocytes within two or three days[1,6,7]. The normal timing of oligodendrocyte development can be restored if embryonic optic-nerve cells are cultured in medium conditioned by type-1 astrocytes[8], the first glial cells to differentiate in the nerve[3]: in this case the progenitor cells continue to proliferate, the first oligodendrocytes appear on the equivalent of the day of birth, and new oligodendrocytes continue to develop over several weeks, just as *in vivo*[7]. Here we show that platelet-derived growth factor (PDGF) can replace type-1-astrocyte-conditioned medium in restoring the normal timing of oligodendrocyte differentiation *in vitro* and that anti-PDGF antibodies inhibit this property of the appropriately conditioned medium. We also show that PDGF is present in the developing optic nerve. These findings suggest that type-1-astrocyte-derived PDGF drives the clock that times oligodendrocyte development.

Table 1 Effect of PDGF on O-2A progenitor-cell proliferation and differentiation into oligodendrocytes *in vitro*

Age of optic nerve	PDGF (ng ml^{-1})	Days in culture	Number of O-2A progenitor cells	Number of oligodendrocytes
E17	0	2	8±5	15±7
E17	0	3	5±3	19±8
E17	2	2	51±8	0
E17	2	3	117±21	0
E17	2	4	214±38	4±2
E18	0	2	8±3	55±7
E18	2	2	135±17	0
E18	2	3	317±45	10±4
E19	0	1	14±2	70±4
E19	2	1	120±9	0
E19	2	2	199±28	2±1
E18	2	2	148±13	0
E18	2	3	287±23	12±6
E18	10	2	133±11	0
E18	10	3	278±37	8±4

Optic nerves from embryonic S/D rats were dissociated into single cells and cultured on poly-D-lysine (PDL)-coated glass coverslips (about 30,000 cells per coverslip) in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, insulin, transferrin, bovine serum albumin, progesterone, putrescine, thyroxine, tri-idodothyronine and 0.5% FCS as described[7]. Purified human PDGF (R and D Systems, Inc.) was added at the start of the culture. After 1–4 days, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (*p*H 7.5) for 5 min at room temperature and stained successively with monoclonal anti-galactocerebroside (GC) antibody[19] (ascites fluid diluted 1:1000), fluorescein-coupled goat anti-mouse IgG3 (G anti-IgG3-Fl, Nordic, diluted 1:100), A2B5 monoclonal antibody[20] (ascites fluid diluted 1:100) and finally rhodamine-coupled goat anti-mouse Ig (G anti-MIg-Rd, Cappel, diluted 1:100); the cells were then post-fixed in acid-alcohol, mounted in glycerol and examined in a Zeiss Universal fluorescence microscope, as described[7]. O-2A progenitor cells were identified by their antigenic phenotype (A2B5$^+$, GC$^-$)[1,15] and characteristic process-bearing morphology[15], while oligodendrocytes were identified as GC$^+$ process-bearing cells[11]. The total numbers of these cells were counted on each coverslip and the results are expressed as means ± s.d. of at least three experiments. The concentration of PDGF required for half-maximal stimulation of O-2A progenitor-cell proliferation was ~0.5 ng ml^{-1} (ref. 9).

We were led to study the effect of PDGF on the timing of oligodendrocyte development *in vitro* by recent evidence that PDGF is a potent mitogen for O-2A progenitor cells in culture[9,10] and that cultured type-1 astrocytes stimulate O-2A progenitor-cell proliferation[8] by secreting PDGF[9]. In the present study, optic nerve cells from embryonic day 17 (E17) Sprague–Dawley (S/D) rats were cultured in medium containing 0.5% fetal calf serum (FCS). As previously reported[7], within two days most of the O-2A progenitor cells in such cultures stopped dividing and differentiated into oligodendrocytes, which were identified by the binding of antibody against galactocerebroside (GC)[11] (Table 1). When human PDGF (R and D Systems, Inc., Minneapolis) was added, however, the O-2A progenitor cells continued to proliferate, doubling in number approximately every day, and the first oligodendrocytes developed after four days, equivalent to the time of birth (Table 1). The same result was obtained if the cells were cultured in type-1-astrocyte-conditioned medium (ACM), as reported previously[7] (data not shown). When E18 or E19 optic nerve cells were cultured in PDGF, the first oligodendrocytes developed after three and two days, respectively, again equivalent to the day of birth (Table 1). The same results were obtained with a fivefold higher concentration of PDGF (Table 1), with human PDGF obtained from Raines and Ross[12], and with porcine PDGF obtained either from R and D Systems or from Stroobant and Waterfield[13] (data not shown).

These results indicate that PDGF can mimic the effects of ACM in restoring in culture the normal timing of oligodendrocyte development observed *in vivo*, raising the possibility that PDGF is the factor in ACM responsible for this activity. To test this possibility, E17 optic nerve cells were cultured in ACM together with an IgG fraction of a goat anti-PDGF antiserum. As shown in Table 2, these antibodies completely blocked the ability of ACM both to stimulate O-2A progenitor-cell proliferation (as reported previously[9]) and to restore the normal *in vivo* timing of oligodendrocyte development in culture (Table 2). The same result was obtained using an IgG fraction of a rabbit antiserum[14] obtained from C.-H. Heldin (data not shown). IgG fractions of goat and rabbit antisera against mouse immunoglobulin, used at the same or tenfold higher concentration, had no such effect (data not shown). The addition of exogenous PDGF together with the anti-PDGF antibodies completely overcame the inhibitory activity of the antibodies (Table 2).

Table 2 Effect of anti-PDGF antibodies on the ability of conditioned medium (ACM or ONCM) to restore the *in vivo* timing of oligodendrocyte development in cultures of E17 optic nerve cells

Conditioned medium	Anti-PDGF antibodies (90 μg ml^{-1})	PDGF (ng ml^{-1})	Number of O-2A progenitor cells	Number of oligodendrocytes
none	−	0	12±5	25±11
none	−	2	71±9	0
ACM	−	0	75±13	0
ACM	+	0	15±7	24±8
ACM	+	15	82±18	0
ONCM	−	0	64±8	0
ONCM	+	0	8±2	31±5

Cells from E17 optic nerves were prepared, cultured for 2 days and stained as in Table 1. Purified type-1 astrocytes from newborn rat cerebral cortex were prepared as described[21]; after growing for several weeks in DMEM supplemented with 10% FCS, the astrocytes were grown in the defined medium (with 0.5% FCS) described in Table 1 for 2 days and the medium was collected as ACM. Newborn optic nerve cells (5×10^5 in 2 ml in PDL-coated 3.5 mm Nunc tissue-culture dishes) were cultured as described[1]; after 1 day in DMEM containing 10% FCS, the cultures were switched to defined medium containing 0.5% FCS, which was collected after 1 day as ONCM. The conditioned media were tested on E17 optic-nerve cultures to find the highest dilution that would still restore the normal timing of oligodendrocyte differentiation, and this concentration (which varied from undiluted to diluted 1:10) was used in these experiments. The medium was changed after 24 h, and fresh conditioned medium, anti-PDGF antibody, and human PDGF (R and D Systems, Inc.) were added. The goat anti-human PDGF antibodies (an IgG fraction prepared by ion-exchange chromatography) were purchased from Collaborative Research Inc. (Bedford, Massachusetts); 50 μg ml^{-1} of the antibody completely neutralized the mitogenic activity of 5 ng ml^{-1} of human PDGF for O-2A progenitor cells (data not shown), and for NIH 3T3 cells (according to the Collaborative Research specification sheet). The results are expressed as means ± s.d. of three separate experiments, except for the results with ONCM where they are triplicates of a single experiment.

The ACM used in the present and previous studies[7–9] was derived from cultures of type-1 astrocytes purified from neonatal-rat cerebral cortex. To determine whether type-1 astrocytes in optic nerve cell cultures also secrete PDGF, we cultured E17 optic nerve cells in medium conditioned over a high density culture of newborn optic nerve cells; almost 60% of newborn optic nerve cells are type-1 astrocytes[3]. As shown in Table 2, such optic nerve conditioned medium (ONCM) kept O-2A progenitor cells dividing and prevented these cells from prematurely differentiating into oligodendrocytes; this activity was

completely inhibited by anti-PDGF antibodies. We have previously provided evidence, however, that the endogenous type-1 astrocytes in E17 optic nerve cultures are too few in number and/or are unable to recover quickly enough from the dissociation procedure to produce sufficient mitogen to keep the progenitor cells dividing and to prevent their premature differentiation[7].

To determine whether PDGF is made in the developing optic nerve, we tested an extract of three-week-old rat optic nerve for its ability to stimulate O-2A progenitor cells in culture to incorporate bromodeoxyuridine (BrdU) into DNA before and after the extract was treated with anti-PDGF antibodies. As shown in Table 3, the extract stimulated progenitor cells to incorporate

Table 3 Stimulation of BrdU incorporation in O-2A progenitor cells by optic nerve extract in culture: the effect of anti-PDGF antibodies

Additives	% O-2A progenitor cells labelled with BrdU
None	3±1
PDGF (1ng ml^{-1})	64±4
Optic nerve extract	50±2
Optic nerve extract treated with anti-PDGF antibodies	14±6

Cells from newborn optic nerves were cultured as in Table 1, except that they were maintained without FCS at 5,000 cells per culture. Bromodeoxyuridine (BrdU, 10 μM; Bochringer) was added after 11.5 h, and the cultures were fixed after 48 h and stained with A2B5 antibody, followed by G anti-MIg-F1 (Cappel, 1:100); they were then treated successively with 2 N HCl (to denature the nuclear DNA[22]), 0.1 M $Na_2B_4O_7$, *p*H 8.5 (each for 10 min at room temperature), monoclonal anti-BrdU antibody[23] (culture supernatant diluted 1:5) and finally with G ani-MIgRd. Optic-nerve extract was prepared from three week-old rats as described[18] and used at 220 μg total protein ml^{-1}. For treatment with anti-PDGF antibodies, the extract (264 μg total protein) was incubated with the IgG fraction of goat anti-human PDGF antiserum (135 μg in 0.7 ml of DMEM; Collaborative Research) for 4 h at 4 °C with continuous rotation; protein A-Sepharose (50 μl of swollen gel, Pharmacia) was added and the mixture was incubated for a further 12 h at 4 °C with continuous rotation and then centrifuged for 1 min in an MSE Micro Centaur. Incubating the extract in normal goat serum and then protein A-Sepharose had no effect on the extract's activity; the extract used in the experiment shown was treated in this way. Anti-PDGF antibodies inhibited the activity of the extract to the same extent when they were added directly (85 μg ml^{-1}) to the cultures (data not shown). The results are expressed as means ± s.e.m. of triplicate cultures.

BrdU, and more than 70% of this mitogenic activity was removed by anti-PDGF antibodies. While this suggests that the major mitogen for O-2A progenitor cells in the optic nerve at this age is PDGF, the finding that not all of the mitogenic activity in the extract was removed by the antibodies, or neutralized when progenitor cells were cultured with the extract in the presence of an excess of anti-PDGF antibody (data not shown), suggests that another mitogen(s) is also present.

Our present and previous results provide compelling evidence that PDGF, secreted by type-1 astrocytes, regulates both the proliferation and the timing of differentiation of O-2A progenitor cells *in vitro*. To summarize the evidence: (1) PDGF stimulates the proliferation of O-2A progenitor cells and prevents them from differentiating prematurely into oligodendrocytes in culture (refs 9, 10, and this study); (2) cultures of type-1 astrocytes purified from cerebral cortex make both PDGF and messenger RNA encoding PDGF A chains[9]; (3) when ACM is fractionated by gel filtration, the mitogenic activity for O-2A progenitor cells is found in the same fractions as radiolabelled PDGF[9]; (4) anti-PDGF antibodies inhibit the ability of ACM and ONCM to stimulate O-2A progenitor cell proliferation *in vitro* (ref. 9, 10 and this study) and to restore the normal *in vivo* timing to oligodendrocyte differentiation (this study).

Table 4 Clonal analysis of oligodendrocyte differentiation in optic nerve cell cultures from 7-day-old rats stimulated by PDGF

Clones	Number of cell divisions: 0	1	2	3	4
a,b,c	1P	2ol			
d,e,f	1P	2P	4ol		
g	1P	2P	4P	8ol	
h	1P	2P	3P,1M	6ol	
i	1P	2P	4P	8P	16ol
j	1P	2P	4P	5P,1ol,1M	11ol

Cells from post-natal day 7 (P7) S/D rats were prepared and studied by time-lapse microcinematography as described[16], except that the cells were maintained in human PDGF (10 ng ml^{-1}) instead of ACM. Proliferation and differentiation of cells in microscopic fields containing 2 to 4 O-2A progenitor cells were followed for one week. The O-2A progenitor cells were identified by their characteristic bipolar morphology[15,16] and migratory behaviour[16], while oligodendrocytes were identified by their multipolar morphology[10,15,16] and lack of motility[16]. As shown in the table, oligodendrocyte differentiation occurred after one to four cell divisions in the ten clones studied, although in every experiment there were still dividing O-2A progenitor cells (belonging to other clones) present in the field at the end of filming. P, O-2A progenitor cells; ol, oligodendrocytes; M, cells that migrated out of the microscopic field and could no longer be followed.

Previous studies have suggested that ACM regulates the timing of oligodendrocyte development *in vitro* by keeping O-2A progenitor cells dividing until an intrinsic clock in the progenitor cell initiates the process that leads to oligodendrocyte differentiation[7,15]. The results of these studies were consistent with the possibility that the clock operates by setting a maximum number of divisions a progenitor cell and its progeny can undergo before differentiating. To test this possibility further we cultured optic nerve cells from seven-day-old rats in the presence of PDGF and followed the fate of individual O-2A progenitor cells and their progeny by time-lapse microcinematography. As described previously, O-2A progenitor cells and oligodendrocytes could be easily recognized as motile bipolar cells and immotile multipolar cells, respectively[16]. In nine of the ten clones studied, all of the descendants of a single progenitor cell differentiated together into non-dividing oligodendrocytes after the same number of cell divisions (Table 4); in the other clone (clone j in Table 4), the progenitor cells differentiated within one cell division of one another. These findings are consistent with previous single-cell experiments and the 'mitotic clock' hypothesis[15] but do not exclude other timing mechanisms.

Whatever the timing mechanism, it is clear that oligodendrocyte differentiation is associated with withdrawal from the cell cycle[4,5,8]. The relationship between the two processes, however, is uncertain. The clock in the progenitor cell might primarily control the onset of oligodendrocyte differentiation, with the cessation of proliferation following as a consequence. Alternatively, the clock might primarily control the onset of unresponsiveness to PDGF, with oligodendrocyte differentiation following as a consequence of withdrawal from the cell cycle. We favour the second possibility as it would most simply explain why O-2A progenitor cells differentiate prematurely when deprived of PDGF (see Table 2).

Whereas oligodendrocyte differentiation seems to be the constitutive pathway of O-2A progenitor cell development, which is automatically triggered when a progenitor cell is deprived of signals from other cells[17] or when the instrinsic timer reaches the appropriate point[7,15], type-2 astrocyte differentiation seems to be induced by a protein signal that greatly increases in concentration in the optic nerve after the first postnatal week[18]. While the mechanisms that control the timing and direction of O-2A progenitor cell differentiation *in vitro* are beginning to emerge, it remains to show that the same mechanisms operate *in vivo*.

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The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria

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Fc receptors on phagocytic cells in the blood mediate binding and clearance of immune complexes, phagocytosis of antibody-opsonized microorganisms, and potently trigger effector functions, including superoxide anion production and antibody-dependent cellular cytotoxicity. The Fc receptor type III (FcγR III, CD16), present in 135,000 sites per cell 1 on neutrophils and accounting for most of FcR in blood, unexpectedly has a phosphatidylinositol glycan (PIG) membrane anchor. Deficiency of FcγR III is observed in paroxysmal nocturnal haemoglobinuria (PNH), an acquired abnormality of haematopoietic cells[2] affecting PIG tail biosynthesis or attachment[3], and is probably responsible for circulating immune complexes[4] and susceptibility to bacterial infections associated with this disease[5]. Although a growing number of eukaryotic cell-surface proteins with PIG-tails are being described[6,7], none has thus far been implicated in receptor-mediated endocytosis or in triggering of cell-mediated killing. Our findings on the FcγR III raise the question of how a PIG-tailed protein important in immune complex clearance *in vivo*[8,9] and in antibody-dependent killing[10] mediates ligand internalization and cytotoxicity. Together with our results, previous functional studies on FcγR III and FcγR II[11,12] suggest that these two receptors may cooperate and that the type of membrane anchor is an important mechanism whereby the functional capacity of surface receptors can be regulated.

Three different types of FcγR have been distinguished in humans using monoclonal antibodies[13] (mAb). FcγR III (CD16) of relative molecular mass (M_r) 50–70,000 (50–70K) is found on neutrophils, large granular lymphocytes, and macrophages, but not on monocytes. FcγR III was first identified with a mAb (3G8) that blocked immune complex binding to neutrophils[1] and subsequently with other mAb of the CD16 cluster[14]. FcγR II (CDw32) is a 40K receptor on neutrophils, monocytes, eosinophils, platelets and B cells[13]. FcγR I is a 72K protein and is found on monocytes[13]. FcγR III and FcγR II have low affinity for monomeric IgG and thus preferentially bind immune complexes by multiple receptor–ligand interactions, whereas FcγR I is sufficiently high affinity to bind monomeric IgG.

Our first evidence that FcγR III is anchored by PIG came from studies on leukocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH). PNH is an acquired defect of haematopoietic precursor cells in either the biosynthesis or the attachment of the PIG tail and may affect clonal progeny in the erythroid, monocytic, granulocytic, and thrombocytic lineages[2,15,16]. Previous studies on erythrocytes and leukocytes from PNH patients have demonstrated a selective deficiency of PIG-tailed proteins, including decay accelerating factor (DAF), acetylcholinesterase, alkaline phosphatase and the PIG-anchored form of lymphocyte function-associated antigen 3 (LFA-3), (refs 3, 6 and 7). The deficiency of DAF accounts for susceptibility of erythrocytes to complement-mediated lysis in PNH. However none of these previously identified deficiencies can explain the occurrence of circulating immune complexes[4] and the 20% and 50% of mortalities caused by bacterial infections and thrombosis respectively[5].

Quantitation of FcγR III expression using immunofluorescence flow cytometry show that it is markedly deficient on PNH neutrophils (Fig. 1*a*). This deficiency was found in all six patients studied (D.E., S.B., J.M., J.E., B.I., C.G.) and results with five different CD16 (FcγR III) mAb were identical. Some patients such as J.E. (Fig. 1*a*, curve 3) showed normal as well as deficient granulocyte clones. Patients showed consistent variation in the extent of deficiency in the abnormal clone. The amount of FcγR III expression on affected cells ranged from 2% (patient D.E.) to 19% (patient J.E.) averaging 7% of normal, perhaps reflecting the degree of penetrance of the acquired defect in PNH. In all cases, deficiency of FcγRIII paralleled deficiency of DAF. In contrast, deficient neutrophils expressed normal levels of HLA-A,B, LFA-1, Mac-1 and FcγR II (CDw32) (Fig. 1*a*). PNH monocytes showed normal expression of FcγR I and II, although they were deficient in DAF (Fig. 1*b*). These results suggested that the neutrophil FcγR III has a PIG tail, whereas the FcγR I and FcγR II do not.

PIG-anchored proteins can be specifically cleaved from cell surfaces with phosphatidylinositol-specific phospholipase C[3,6,7] (PIPLC). We therefore investigated the susceptibility of Fc receptors to PIPLC (Table 1). PIPLC released 75-84% of the cell surface FcγR III and DAF from healthy granulocytes, while FcγR II, HLA-A,B and LFA-1 were unaffected. On monocytes, PIPLC released 84% of DAF whereas it had no effect on FcγR II, FcγR I, HLA-A,B and LFA-1. Results with PIPLC prepared from *S. aureus* and *B. thuringiensis* were identical and show that FcγR III on neutrophils, but not FcγR II on the same cells or FcγR I and II on monocytes, have PIG anchors. Lack of a PIG anchor on FcγR II is consistent with the prediction (from cDNA sequence[17]) that it possesses a transmembrane domain and a 76 residue hydrophilic cytoplasmic tail.

CHAPTER

10

Progenitor Cells of the Adult Human Subcortical White Matter

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OLIGODENDROCYTE PROGENITOR CELLS OF THE ADULT MAMMALIAN BRAIN

Neural Progenitor Cells of the Adult Brain

Over the past few decades, historic notions of the structural immutability and cellular constancy of the adult vertebrate brain have been largely dispelled. Neurogenesis was first demonstrated in the rodent olfactory bulb and the hippocampus (Altman and Das, 1965, 1966; Kaplan and Hinds, 1977; Kaplan, 1985) and the songbird vocal control centers (Goldman and Nottebohm, 1983; Nottebohm, 1985). The phenomenon of adult neurogenesis has now been described throughout vertebrate phylogeny (Goldman, 1998), including monkeys (Gould *et al.*, 1998) and humans (Eriksson *et al.*, 1998; Kirschenbaum *et al.*, 1994; Pincus *et al.*, 1998). In all species yet examined, newly generated neurons seem to be generated from multipotential stem cells, the principal source of which appears to be the periventricular subependyma (SVZ) (Goldman *et al.*, 1993; Lois and Alvarez-Buylla, 1993). In addition, restricted pools of mitotically competent but phenotypically biased neuronal progenitor cells appear to derive from these stem cell populations. These neuronally restricted pools include the anterior subventricular zone of the forebrain and its rostral extension through the olfactory subependyma, as well as the subgranular zone of the hippocampus, each of which give rise almost exclusively to neurons *in vivo*. However, persistent multipotential stem cells have been reported in cultures derived from each of these regions (Gage *et al.*, 1998), suggesting that the apparent neuronal restriction of these progenitor populations may reflect not the inherent lineage capacity of the cells, so much as local environmental signals biasing toward neuronal differentiation (Seaberg and van der Kooy, 2002).

Besides these persistent neuronal progenitors and multipotential neural stem cells, more restricted lineages of glial progenitor cells also persist in the adult brain, in both the residual ventricular zone (Levison and Goldman, 1993; Luskin, 1993), as well as dispersed throughout the subcortical and cortical parenchyma (Gensert and Goldman, 1996; Levine *et al.*, 2001; Noble, 199 ; Reynolds and Hardy, 1997). Indeed, in contrast to the restricted distribution of neuronal progenitor cells and SVZ stem cells, oligodendrocyte progenitor cells (OPCs) seem to be extraordinarily widespread in the adult mammalian brain.

Oligodendrocyte Progenitors of the Normal Adult Rodent Brain

The principal class of OPCs in adult rodents is a bipotential astrocyte-oligodendrocyte progenitor cell designated the O-2A progenitor, by virtue of its generation *in vitro* of oligodendrocytes and type 2 astrocytes, the latter comprising the traditionally recognized fibrous astrocytes of the white matter. These cells were initially isolated from the optic nerves of perinatal rats, as O-2A progenitors (Raff *et al.*, 1983b). In neonatal rats, OPCs are characterized by expression of the GD3 and GQ gangliosides, the latter recognized by the monoclonal antibody A2B5, which has been used to identify this cell population (Noble *et al.*, 1992). Though similar progenitors were long ago reported in the adult optic nerve (Vaughn, 1969), the isolation of adult OPCs, or O-2A^{Adult} progenitors, was only accomplished relatively recently in rodents (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). These cells have since been isolated from the adult rat ventricular zone, spinal cord, cerebellum, and subcortical white matter (Engel and Wolswijk, 1996; Gensert and Goldman, 1996; Levine *et al.*, 1993).

Antigenic Recognition of Adult OPCs

Little is known about the natural history of the adult OPC in normal adults. In histological sections of the adult rodent brain, OPCs have mainly been identified by their expression of both NG2 chondroitin sulfate proteoglycan (Levine *et al.*, 1993; Nishiyama *et al.*, 1997) and the platelet derived growth factor-alpha receptor (PDGF-αR). The expression of PDGF-αR and the NG2 epitope substantially overlaps in rats (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992). Moreover, a persistent population of O4/NG2 co-expressing cells has been demonstrated in the adult rat cerebral cortex, effectively bridging the antigenic gap between early and committed OPCs (Reynolds and Hardy, 1997). On the basis of these studies, NG2-immunoreactivity has been developed as a surrogate marker for parenchymal oligodendrocyte progenitor cells. In addition, adult-derived OPCs have several features that may allow them to be distinguished: Whereas the perinatal OPC utilizes vimentin as an intemediate filament and does not express the oligodendrocytic sulfatide recognized by Mab O4, its adult counterpart does not express vimentin, but does express O4 (Shi *et al.*, 1998; Wolswijk and Noble, 1989; Wolswijk *et al.*, 1991). These parenchymal OPCs are present in both gray and white matter, and exist *in vivo* as extensively branched cells. The NG2 population represents as many as 5–8% of all the cells in the adult rodent brain (Dawson *et al.*, 2000); this is congruent with earlier estimates that 5% of all glia in the optic nerve may be progenitors (Vaughn and Peters, 1968).

Turnover

OPCs in the adult brain may include both slowly dividing cells in normal parenchyma and a quiescent cell population that responds only to injury or demyelination. *In vivo* studies of the adult cerebellar cortex reveal the presence of slowly dividing OPCs with a mitotic index of 0.2 to 0.3% (Levine *et al.*, 1993). Nevertheless, OPCs seem to constitute the main cycling population of the adult brain parenchyma. Bromodeoxyuridine (BrdU) labeling of the intact spinal cords of 13- to 14-week-old rats has shown that 10% of all cells in the white matter incorporated BrdU, of which 70% expressed NG2. In animals maintained for 4 weeks after BrdU injection, BrdU-labeled astrocytes and oligodendrocytes were noted, indicating that the cycling NG2 cells would have generated both cell types (Horner *et al.*, 2000). In studies using retroviral labeling to mark dividing cells, 35% of the cycling cells in the adult cortex co-labeled with NG2, and these were distinctly present as clusters. Furthermore, these NG2-positive clusters doubled in size every 3 months (Levison *et al.*, 1999). Using similar retroviral labeling techniques, the presence of cycling cells that preferentially give rise to oligodendrocytes has been shown in both the subventricular zone (SVZ) and subcortical white matter of adult rats (Gensert and Goldman, 1996; Levison and Goldman, 1993).

Lineage Potential

Previous studies had concluded that the perinatal OPC has a limited life span *in vivo*, which was attributed to a pattern of "exhaustive" symmetrical division and differentiation in

oligodendrocytes (Temple and Raff, 1986). Yet OPCs now appear to be maintained throughout life. This suggests that at least a fraction of OPCs may arise through a self-renewing, asymmetrical divisions, such that OPCs generate both differentiated progeny and themselves (Wren *et al.*, 1992). Indeed, adult OPCs of both rodents and humans retain their ability to generate oligodendrocytes and astrocytes over several generations *in vitro* (Tang *et al.*, 2000).

It seems likely that perinatal OPCs are the source of adult OPCs. Using time-lapse microcinematography, it has been shown that "founder cells" exhibiting properties of perinatal OPCs eventually give rise to cells with the properties of adult OPCs (Wren *et al.*, 1992). As noted, just as repetitive passage of perinatal OPCs gives rise to cells with adult OPC-like properties (Wolswijk *et al.*, 1990), slowly dividing adult OPCs can respond to FGF and PDGF by assuming the more rapid expansion kinetics typical of perinatal OPCs. Together, these data argue that perinatal and adult OPCs constitute two points along the differentiation spectrum of a common lineage. Nonetheless, diversification within that lineage may nonetheless have resulted in substantial phenotypic heterogeneity among adult OPCs (Gensert and Goldman, 2001).

Humoral Control of Oligoneogenesis

Adult and perinatal OPCs share many commonalities in their responses to humoral growth factors, but nonetheless exhibit differential responses to both neural mitogens and differentiation agents. These include, but are by no means limited to, the following:

1. Platelet derived growth factor. PDGF is perhaps the most prominent described oligotrophin and has been implicated in both the mitotic expansion of OPCs and their initiation of terminal lineage commitment (Hart *et al.*, 1989a; Noble *et al.*, 1988; Raff *et al.*, 1988; Wolswijk *et al.*, 1991). OPCs uniquely express high levels of PDGFα receptor, and can be specifically identified on that basis (Ellison and de Vellis, 1994; Fruttiger *et al.*, 1999; Hart *et al.*, 1989b). In response to PDGF, both perinatal and adult OPCs enter the mitotic cycle. However, cycling time differs in the two cell populations, in that adult OPCs have a slow, 3- to 4-day cell cycle, whereas perinatal OPCs divide daily (Noble *et al.*, 1988); (Wolswijk *et al.*, 1991). In OPCs derived from the adult spinal cord, PDGF alone supports the slow mitotic expansion of OPCs, as the cells divide slowly and undergo asymmetrical division, generating a differentiated oligodendrocyte and another progenitor (Engel and Wolswijk, 1996). However, in the presence of PDGF and FGF, adult OPCs accelerate their cycle progression, dividing rapidly and apparently symmetrically to yield additional progenitors. They then assume the bipolar morphology and A2B5 immunoreactivity of oligodendrocyte progenitor cells, but fail to generate oligodendrocytes without downstream inductive differentiation. As a corollary to this "perinatalization" of adult-derived OPCs, cultures of perinatal OPCs expanded over long periods of time in the presence of PDGF alone develop the cyclicity of adult OPCs (Tang *et al.*, 2000). These results suggest that in rodents at least, perinatal and adult-derived OPCs represent points on a continuum of differentiative state, rather than discrete phenotypes

2. Fibroblast growth factor. FGF differentially regulates OPC proliferation and differentiation in culture and modulates gene expression of its own receptors in a developmental and receptor type-specific manner (Bansal *et al.*, 1996). Most *in vitro* studies show that bFGF is a major mitogen for cells in the oligodendrocyte lineage (Besnard *et al.*, 1989; Eisenbarth *et al.*, 1979). It has been shown to stimulate the proliferation of late progenitors and inhibit their terminal differentiation (Bansal and Pfeiffer, 1994; McKinnon *et al.*, 1990). More important, it establishes the responsiveness to PDGF by up-regulating the expression of PDGF-αR (McKinnon *et al.*, 1990). Most studies with adult OPCs show that bFGF is most mitogenic when used in combination with PDGF (Mason and Goldman, 2002; Tang *et al.*, 2000). Recently it has been shown that OPCs maintained in the presence of bFGF eventually become resistant to replicative senescence (Tang *et al.*, 2001). Besides its well-documented effect on OPCs, bFGF also induces the down-regulation of myelin genes, such as myelin basic protein (MBP), in mature oligodendrocytes without reverting

them to the progenitor phenotype or effecting reentry into the cell cycle (Bansal and Pfeiffer, 1997; Grinspan *et al.*, 1993).

3. Neurotrophin-3 (NT3). Whether NT3 has proliferative or differentiative effect on OPCs is yet unresolved. One study indicated that NT3, specifically in combination with PDGF, is proliferative for post-natal OPCs both *in vitro* and *in vivo* (Barres *et al.*, 1994b). Other studies, however, found that NT3 is not proliferative for adult OPCs alone or in combination with PDGF and bFGF (Engel and Wolswijk, 1996; Ibarrola *et al.*, 1996). Perhaps this differential response may be a function of the different OPC-types that have been used for the two studies. In the contused adult spinal cord, NT3 has been shown to increase OPC proliferation and myelination (McTigue *et al.*, 1998). A recent *in vitro* study with OPCs from adult spinal cord dissociates indicates that NT3 induced myelination and the proliferation of O4+/O1-cells (Yan and Wood, 2000).

4. Neuregulin. The neuregulins are a family of soluble and transmembrane protein isoforms, of which glial growth factor 2 (GGF2) is a member (Adlkofer, 2000). The neuregulins act upon erbB receptors, in particular on the erbB2, 3, and 4 heterodimeric receptors (Buonanno and Fischbach, 2001). Perinatal OPCs divide in response to GGF provided cAMP levels are high, so that adenyl cyclasae and erbB stimulation may operate synergistically as glial progenitor mitogens (Shi *et al.*, 1998). Canoll *et al.* observed a similar proliferative effect on $O4^+/O1^-$ progenitors (Canoll *et al.*, 1996). Adult OPCs respond to GGF2 as well, although their mitogenic activation by GGF2 appears to require the concurrent activation of the PDGF receptor, along with elevated cAMP. An interesting feature of neuregulins includes their induction of phenotypic reversion by differentiated oligodendrocytes (Canoll *et al.*, 1999). OPCs produce neuregulins (Raabe *et al.*, 1997) as well as respond to it (Shi *et al.*, 1998). Since they express full-length neuregulin erbB receptors, OPCs may utilize neuregulins as an autocrine factor, as well as a neuronally derived oligotrophin (Fernandez *et al.*, 2000). This is likely to obtain in the environment of the adult human white matter, from which oligodendrocytes have similarly been shown to produce neuregulins and express receptors to them (Cannella *et al.*, 1999; Deadwyler *et al.*, 2000).

5. Triiodothyronine. When OPCs derived from optic nerves or cerebral hemispheres are cultured in the presence of T3, they immediately stop dividing and differentiate into oligodendrocytes. In fact, the number of times an OPC can divide varies inversely with its concentration of T3, implicating T3 as an oligodendrocytic differentiation factor (Baas *et al.*, 1997). T3 seems to play a major role in controlling the timing of OPC differentiation (Barres *et al.*, 1994a). Accordingly, hypothyroid states have been associated with deficits in early myelination in neonatal cretinism, which may reflect a failure in T3-mediated OPC expansion.

6. Insulin growth factor-1 (IGF-1). During development, high levels of IGF1 are observed just before active myelination commences (Bach *et al.*, 1991; Carson *et al.*, 1993). IGF-1 increases proliferation and survival, enhance differentiation, and modulate the expression of MBP in both OPCs and oligodendrocytes (Barres *et al.*, 1992; McMorris and Dubois-Dalcq, 1988; Saneto *et al.*, 1988).

Oligodendrocyte Progenitors of the Adult Human Brain

The earliest evidence that the adult human brain harbors oligodendrocyte progenitors came from early studies of MS lesions. Histopathologically, these lesions were found to harbor regions of extensively remyelinated axons, as well as numerous free oligodendrocytes (Moore *et al.*, 1985; Prineas and Connell, 1979; Prineas *et al.*, 1984). Subsequent studies identified populations of immature cells expressing the neural carbohydrate epitope HNK1; these were postulated to comprise early oligodendroglia, although these early studies were unable to identify any definitive oligodendrocyte progenitor cell phenotype (Prineas *et al.*, 1989; Wu and Raine, 1992).

PDGF-αR expressing OPCs have been shown in both MS lesions and surrounding normal white matter (Scolding *et al.*, 1998). These PDGF-αR^+ cells were found to be more

frequent in or near MS lesions compared to normal surrounding white matter (WM), and those near lesions were more often cycling, as revealed by immunoreactivity for Ki67, a marker of proliferation (Maeda *et al.*, 2001). Corroborating these observations with another marker of phenotype, the NG2 chondroitin sulfate proteoglycan was demonstrated in both normal adult human WM and MS lesions. As in their rodent counterparts, human $NG2^+$ cells were found to be extensively ramified. Cells morphologically similar to $NG2^+$ cells were reported to express PDGF-αR as well, although co-expression of the two by a common phenotype has yet to be directly demonstrated.

Premyelinating oligodendrocytes—defined by their expression of proteolipid protein (PLP), and their contiguity with axons despite an absence of attendant ensheathment—have also been shown in such MS lesions (Chang *et al.*, 2002). Interestingly, $NG2^+$ cells are virtually absent from lesions lacking premyelinating oligodendrocytes. This suggests that $NG2^+$ cells might be the source of these premyelinating oligodendrocytes. However, the NG2 chondroitin sulfate may not be specific to OPCs in the adult human brain, as microglial cells express or sequester high levels of NG2-IR (Pouly *et al.*, 1999; also Nunes, Roy, and Goldman, unpublished observations). Indeed, in dissociates of both fetal and adult human brain tissue, most $NG2^+$ cells were microglial (Pouly *et al.*, 1999). To establish a more reliable marker of OPCs in adult human tissues, Scolding *et al.* thus assessed the phenotypic specificity of two cardinal markers of OPC phenotype in rodents, specifically the PDGF-α receptor and the A2B5 epitope represented by the GQ ganglioside. By scoring the incidence of both $PDGF\text{-}\alpha R^+$ and $A2B5^+$ cells in tissue print preparations of adult human white matter, Scolding and colleagues determined that these markers recognize a common parenchymal progenitor cell population. On this basis, they were able to report the first estimates of the incidence of oligodendrocyte progenitor cells in the human white matter (Scolding *et al.*, 1999).

Despite this wealth of histological assessment of parenchymal progenitor cells, relatively few studies have yet correlated the antigenic expression patterns of single parenchymal phenotypes with their lineage potential, either *in vivo* or *in vitro*. As a result, it remains unclear if the expression of markers such as GD3, NG2, A2B5, or PDGF-αR is specific to adult OPCs, or whether it instead is shared among different, already discrete lineages at similarly early points in their phenotypic specification. The uncertain lineage potential of histologically antigen-defined oligodendrocyte progenitor cells has derived in part from an historic inability to identify or isolate these cells from human brain tissues. An early attempt to identify oligodendrocyte progenitors in dissociates of adult human brain (Kim *et al.*, 1983) was followed by successful *in vitro* and *in vivo* demonstrations of immature oligodendroglia, which were termed pro-oligodendrocytes because of their post-mitotic state. These cells were defined as being $O4^+/A2B5^-/GalC^-$ (Armstrong *et al.*, 1992). Pro-oligodendrocytes were further characterized and found to express the PDGF-αR in tissue, where they were estimated to constitute 2% of the total cell population (Gogate *et al.*, 1994). Subsequent studies of the adult human white matter *in vitro* revealed the presence of mitotic cells that could give rise to oligodendrocytes, though the identity of the precursor remained unclear (Roy *et al.*, 1999; Scolding *et al.*, 1995);.

Humoral Control of Adult Human Oligodendrocyte Progenitor Cells

Human and rodent OPCs differ not only in their antigenic expression patterns, as noted, but also as in their responses to humoral growth factors. Adult human OPCs do not proliferate in response to bFGF, PDGF, or IGF-1, each of which can act singly as a mitogen for rodent OPCs (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Prabhakar *et al.*, 1995). Instead, in human OPCs, IGF-1 has been shown to increase the proportion of post-mitotic pro-oligodendrocytes and to promote the maturation of these cells as oligodendrocytes (Armstrong *et al.*, 1992). Human OPCs also seem to be mitotically unresponsive to astrocyte conditioned medium (Armstrong *et al.*, 1992; Gogate *et al.*, 1994; Prabhakar *et al.*, 1995; Scolding *et al.*, 1995). As noted previously, neuregulin supports the expansion of OPCs and is released by neurons in an activity-dependent manner that might allow the activity-dependent modulation of OPC expansion (Canoll *et al.*, 1996). However, these

observations have yet to be verified as operative in human OPCs. Indeed, little data are available on the factor responsiveness of human OPCs, despite the overt clinical importance of establishing the optimal expansion and differentiation conditions for these cells. Rather, the study of their growth factor responsiveness, patterns of receptor expression, and likely paracrine interactions with other parenchymal cell populations have been impeded by the inability to identify and isolate OPCs from the adult human brain, and hence the lack of material for molecular and cellular analysis.

Isolation of Adult Human Oligodendrocyte Progenitor Cells

To address the need for isolating enriched populations of adult OPCs, we used promoter-specified fluorescent activated cell sorting (FACS) to identify and extract these cells from adult human brain tissue. Traditionally, FACS has been used to sort live cells on the basis of surface antigen expression, particularly in the hematopoetic system, in which FACS has been used to define and isolate the major stem cell and intermediate progenitor phenotypes generated during lymphopoesis and hematopoiesis. However, the application of FACS to the nervous system was stymied by the lack of identified surface antigens specific to stage or phenotype among neural cells. Yet in 1994, the green fluorescence protein was first identified as a live cell reporter of gene expression (Chalfie *et al.*, 1994). By placing GFP under the transcriptional control of promoters regulating the expression of cell-specific genes, we were able target specific cell phenotypes for FACS isolation. We first applied this approach to extracting neuronal progenitor cells from the fetal ventricular zone (Wang *et al.*, 1998), by transducing ventricular zone cells with GFP placed under the control of the Tα1 tubulin promoter, an early neuronal regulatory sequence (Gloster *et al.*, 1994; Miller *et al.*, 1987, 1989). This approach has since allowed us to isolate neuronal progenitor cells from both the adult human ventricular zone (VZ) and hippocampus (Roy *et al.*, 2000a, 2000b). In addition, by modifying our choice of promoters to those specifically active in even earlier neural progenitors, we were able to isolate less committed neural stem cells from both the adult and fetal human brain (Keyoung *et al.*, 2001; Roy *et al.*, 2000a, 2000b).

The development of promoter-based FACS gave use the means to identify and then isolate oligodendrocyte progenitor cells from the adult human brain (Fig. 10.1). To this end, we used the early promoter for an early oligodendrocyte protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Scherer *et al.*, 1994; Vogel and Thompson, 1988). CNP protein is the earliest myelin-associated protein known to be expressed in developing oligodendrocytes. It is expressed by oligodendrocytes at all ontogenetic stages (Sprinkle, 1989; Weissbarth *et al.*, 1981), including by newly generated cells of oligodendrocytic lineage within the subventricular zone and their mitotic precursors (Scherer *et al.*, 1994; Yu *et al.*, 1994). The 5' regulatory region of the CNP gene includes two distinct promoters, P2 and P1, which encode for two CNP isoforms, CNP1 (46kDA) and CNP2 (48 kDa). These promoters are sequentially activated during development, with the more upstream P2 promoter (P/CNP2) directing transcription to immature oligodendrocytes and their progenitors (Gravel *et al.*, 1998; O'Neill *et al.*, 1997). On this basis, P/CNP2 was chosen to identify oligodendrocyte progenitors from adult human subcortical white matter (Roy *et al.*, 1999). P/CNP2:hGFP was transfected into dissociate of adult human white matter, and following GFP expression 3 to 4 days later, the P/CNP2:GFP$^+$ cells were isolated by FACS (Roy *et al.*, 1999). These cells, maintained in serum-deficient media supplemented with FGF2, PDGF, and NT-3, were bipolar, immunoreactive for A2B5, incorporated BrdU from their culture media, and developed into O4$^+$ oligodendrocytic *in vitro* (Fig. 10.2). These data indicated that the P/CNP2:hGFP-defined cells were mitotic oligodendrocyte progenitors. On this basis, P/CNP2:hGFP$^+$ oligodendrocyte progenitors were extracted directly from adult human WM dissociates using FACS. We found that an average of 0.5 $\pm$ 0.1% of all white matter cells directed P/CNP2:hGFP expression. Given a transfection efficiency of 13.5%, determined using the percentage of GFP expressing cells obtained with p/CMV:GFP for noncell type specific transfection, it could be estimated that over 4% of adult human subcortical WM are P/CNP2-defined progenitors. Immediately after FACS, these P/CNP2:hGFP-separated cells were initially bipolar, and

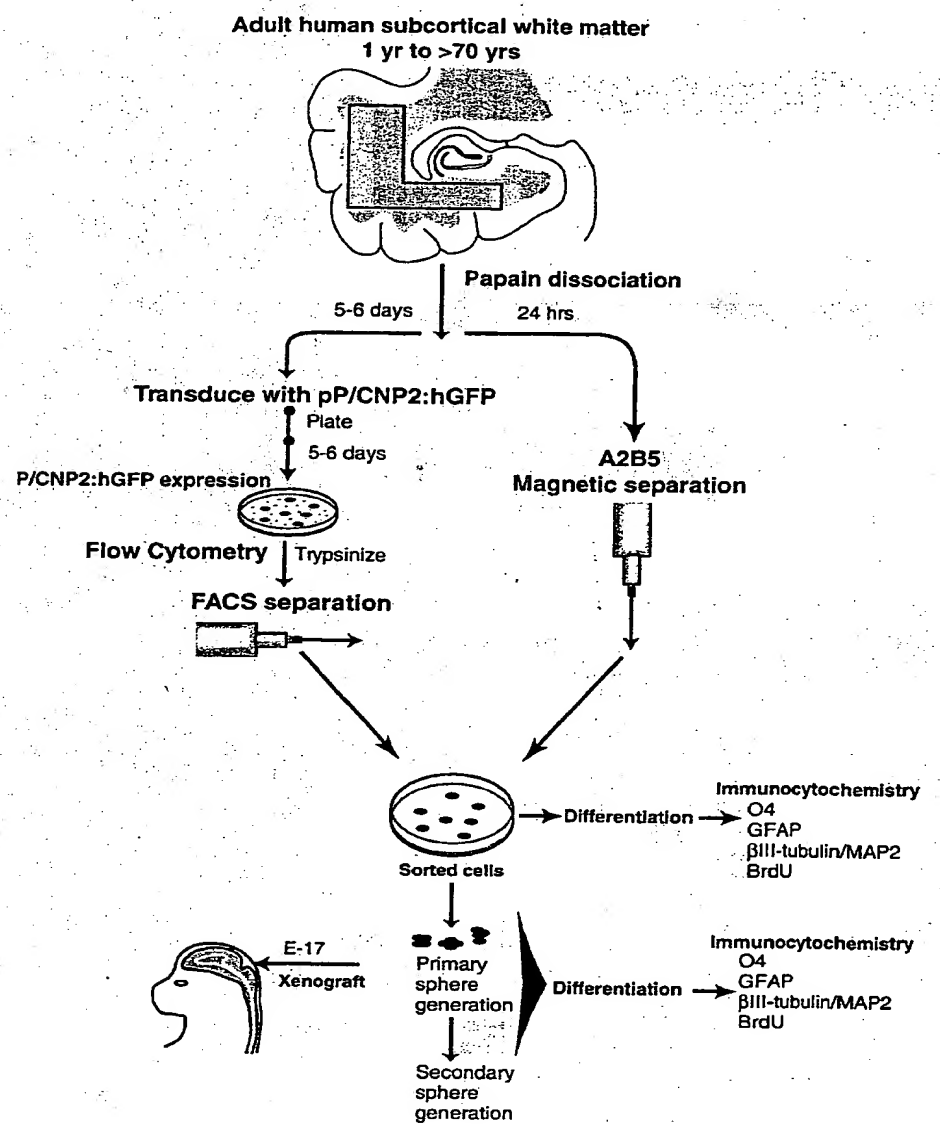

FIGURE 10.1
Oligodendrocyte progenitor cells may be specifically targeted and isolated from the white matter. This schematic outlines basic strategies for isolating oligodendrocyte progenitor cells from the adult white matter, using either fluorescence-activated cell sorting (FACS) or a higher-yield, less specific alternative immunomagnetic isolation (MACS).

expressed the early oligodendrocytic marker A2B5, but none of the differentiated markers O4, O1, or galactocerebroside; over half incorporated BrdU. When followed up to a month in culture, >80% of the PCNP2:hGFP$^+$ cells become oligodendrocytes, progressing through a succession of A2B5, O4, and galactocerebroside expression, recapitulating the developmental sequence of antigenic expression (Noble, 1997). Thus, with this strategy not only was the existence of oligodendrocyte progenitors established in adult human white matter, but a method was developed to separate the progenitors in a form appropriate for engraftment and further analysis.

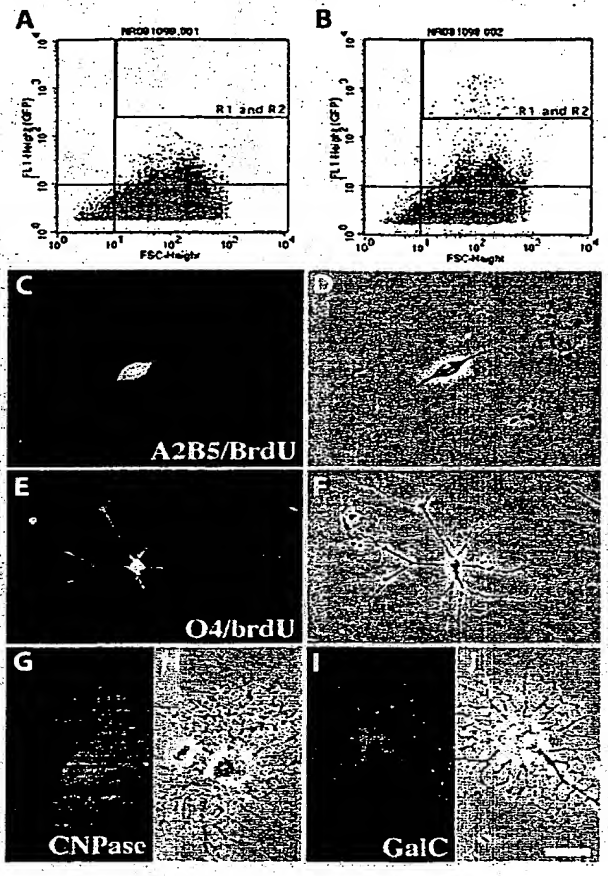

FIGURE 10.2
Sorted human white matter progenitor cells typically mature as oligodendrocytes. (A–B) A representative sort of a human white matter sample, derived from the frontal lobe of a 42-year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity plotted against forward scatter (a measure of cell size). Part A indicates the sort obtained from a nonfluorescent P/hCNP2:*lacZ*-transfected control, while part B indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. (C–D) A bipolar $A2B5^+/BrdU^+$ cell, 48 hours after FACS. (E–F) By 3 weeks post-FACS, P/CNP2:hGFP-sorted cells developed multipolar morphologies and expressed oligodendrocytic O4 (red). These cells often incorporated BrdU, indicating their *in vitro* origin from replicating $A2B5^+$ cells. (G–I) Matched phase (G, I) and immunofluorescent (H, J) images of maturing oligodendrocytes, 4 weeks after P/CNP2:hGFP-based FACS. These cells expressed both CNP protein (H) and galactocerebroside (J), indicating their maturation as oligodendrocytes. Scale bar = 20 μm. Taken from Roy *et al.*, 1999; with permission.

Antigenicity of Oligodendrocyte Precursor Cells

As described earlier, virtually all P/CNP2:hGP-defined OPCs are immunoreactive for A2B5 (Roy *et al.*, 1999). This permitted us to use A2B5-based sorting to increase the yield of isolated progenitors, to numbers sufficient for experimental transplantation. Although both immature neurons and glia express A2B5-immunoreactivity during development (Aloisi *et al.*, 1992; Eisenbarth *et al.*, 1979; Lee *et al.*, 1992), the adult subcortical parenchyma is relatively devoid of young neurons, allowing A2B5 to be used as a selective marker of glial and oligodendrocyte progenitor cells (Raff *et al.*, 1983a; Satoh *et al.*, 1996; Scolding *et al.*, 1999). The specific use of A2B5 as an antigenic surrogate for P/CNP2:hGFP-defined OPCs has thus constituted a significant practical advance. By extracting OPCs via A2B5-based surface-antigen based sorting, the limitations of transfection-based tagging, which include

direct cytotoxicity as well as low efficiency, can be avoided entirely. As a result, the practical issue of acquiring sufficient numbers of viable OPCs to permit transcriptional and biochemical analysis, as well as engraftment studies, can now be effectively addressed.

Multipotential Progenitors of the Adult Human White Matter

Like their lower species counterparts, human OPCs may not be strictly dedicated or autonomously programmed to oligodendrocytic differentiation. When purified from adult human subcortical tissue, derived from surgically resected temporal lobe, white matter progenitor cells (WMPCs) give rise largely to oligodendrocytes. However, when grown under conditions of very low density, we noted that these cells also generate occasional neurons (Roy *et al.*, 1999). On this basis, we asked whether the white matter progenitor cells of the adult human brain might actually constitute a type of multipotential neural progenitor or neural stem cell. We found that white matter progenitor cells, purified by FACS from the adult human brain, can indeed generate neurons as well as both major glial cell types—astrocytes and oligodendrocytes—when raised in culture under conditions of high purity and low density (Nunes *et al.*, 2003). Under these conditions, the cells are effectively removed from other cells, as well as from the proteins that other cells may secrete. Under these conditions, the sorted progenitor cells divide and expand as multipotential clones that generate neurons as readily as oligodendrocytes (Fig. 10.3). They can continue to divide and expand for several months in culture, dividing to increase their

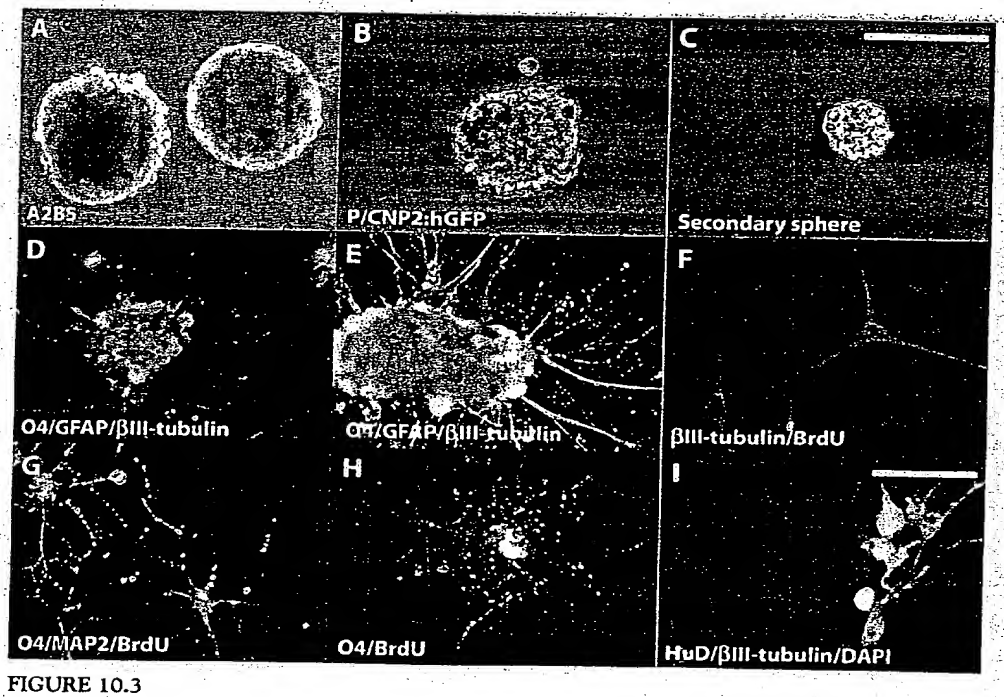

FIGURE 10.3
Adult human WMPCs give rise to multipotential neurospheres. (A) First-passage spheres generated from A2B5-sorted cells 2 weeks post-sort. (B) First-passage spheres arising from P/CNP2:hGFP sorted cells, 2 weeks. (C) Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. (D) Once plated onto substrate, the primary spheres differentiated as βIII-tubulin$^+$ neurons (red), GFAP$^+$ astrocytes (blue), and O4$^+$ oligodendrocytes (green). (E) Neurons (red), astrocytes (blue), and oligodendrocytes (green) similarly arose from spheres derived from P/CNP2:GFP-sorted WMPCs. (F–H) BrdU incorporation (blue) revealed that new neurons (F: βIII-tubulin in red; G: MAP2 in red) and oligodendrocytes (H: O4 in green) were both generated *in vitro*. (I) βIII-tubulin$^+$ neurons (green) co-expressed neuronal Hu protein (Barami *et al.*, 1995; Marusich *et al.*, 1994) (red, yielding; yellow double-label). Nuclei counterstained with DAPI (blue). From Nunes *et al.* (2003). Scale: A–E, 100 μm; F–I, 24 μm.

numbers in the process. Moreover, upon xenograft to the developing fetal rat forebrain, adult human WMPCs can mature into neurons as well as oligodendrocytes and astrocytes *in vivo*, in a region- and context-dependent manner (Fig. 10.4). The nominally glial progenitor cell of the adult human white matter thus appears to constitute a multipotential neural progenitor. These cells appear to be typically restricted by their local brain environment to produce only oligodendrocytes and some astrocytes, in response to local environmental signals whose identities remain to be established. But when removed from the

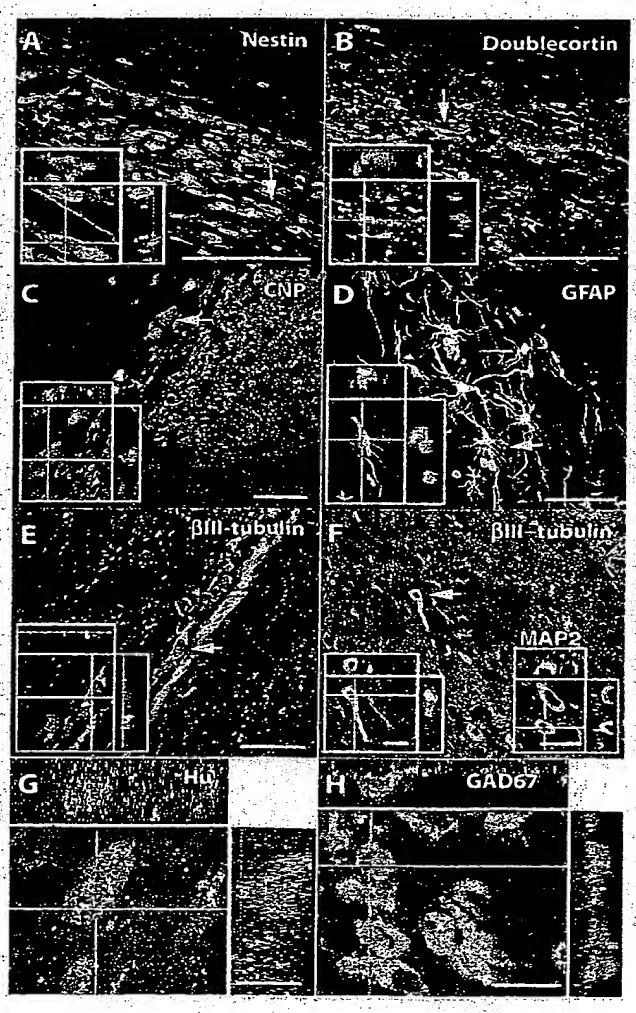

FIGURE 10.4

WMPCs engrafted into fetal rats gave rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and sacrificed a month after birth. These cells were maintained in culture for 10 days prior to implant. (A–B) Nestin$^+$ (red) progenitors and doublecortin$^+$ (red) migrants, respectively, each co-expressing human nuclear antigen (hNA, green) in the hippocampal alvius. (C) CNP$^+$ oligodendrocytes (red) that were found exclusively in the corpus callosum. (D) A low-power image of GFAP$^+$ (green, stained with anti-human GFAP) astrocytes along the ventricular wall. (E) βIII-tubulin$^+$ (green)/hNA$^+$ (red) neurons migrating in a chain in the hippocampal alvius. (F) βIII-tubulin$^+$ and MAP2$^+$ (inset in part F) neurons in the striatum, adjacent to the RMS (antigens in green; hNA in red; yellow: double-stained human nuclei). (G) An Hu$^+$/hNA$^+$ neuron in the septum. (H) An hNA$^+$ (green)/GAD-67$^+$ (red) striatal neuron. Insets in each figure show orthogonal projections of a high-power confocal image of the identified cell (arrow). From Nunes *et al.* (2003). Scale: A–E, 40 μm; F–H, 20 μm.

environment of the brain and from other brain cells, these cells proceed to make all brain cell types, including neurons and glia, and remain able to do so for long periods of time in culture.

This observation has precedent in lower species. Progenitor cells capable of giving rise to multiple lineages, including oligodendrocytes and neurons, have been consistently derived from the cortical and subcortical parenchyma as well as from the ventricular zone of embryos (Davis and Temple, 1994; Qian *et al.*, 1997; Williams *et al.*, 1991). Similar multipotential progenitors have shown to exist in early postnatal rat cortex (Marmur *et al.*, 1998). A more recent study suggested that postnatal rat optic nerve derived O-2A progenitor cells could be "reprogrammed" to multipotential stem cells capable of generating neurons (Kondo and Raff, 2000). This was achieved by sequential exposure of O-2A progenitors to serum to induce astrocytic differentiation, followed by their expansion in the presence of bFGF in serum-free conditions. Constant mitogenic stimulation of adult rat forebrain parenchymal cells with FGF2 has been shown to result in the generation of neurons as well as astrocytes and oligodendrocytes (Palmer *et al.*, 1995; Richards *et al.*, 1992). Together, these observations of the multilineage potential of CNS glial progenitors suggest that the apparent lineage commitment of progenitors might depend on epigenetic factors. As a result, the nominally glial progenitors of the adult white matter may retain far more lineage plasticity and competence than traditionally appreciated. Adult subcortical P/CNP2:hGFP$^+$ progenitors, though competent to generate multiple cell types, may therefore be restricted to the oligodendrocytic lineage by virtue of the epigenetic bias imparted by their environment before their isolation.

A corollary of the environmental restriction of WMPC phenotype is that other, non-white-matter-derived neural progenitors might similarly restrict to oligodendrocytic lineage when presented to the environment of the adult white matter. Indeed, several groups have reported that EGF-expanded murine neural stem cells differentiate as oligodendrocytes upon xenograft (Mitome *et al.*, 2001); remarkably, in none of these models were substantial numbers of oligodendrocytes generated *in vitro*. Similarly, v-myc transformed neural stem cells transplanted to perinatal mice can differentiate as oligodendrocytes once recruited to the white matter (Yandava *et al.*, 1999), but not otherwise, and never *in vitro*.

The Distribution and Heterogeneity of White Matter Progenitor Cells

The persistence and sheer abundance of WMPCs in the adult human brain is striking: Over 3% of the white matter cell population may be sorted on the basis of CNP2:GFP-based FACS, and over half of these cells are mitotically active upon isolation (Roy *et al.*, 1999). That being said, the extent to which this parenchymal progenitor cell population is homogeneous remains unclear; by limiting dilution analysis, only 0.2% of its cells are multipotential (Nunes *et al.*, 2003). Nonetheless, the very existence of multipotential progenitors scattered throughout the white matter parenchyma forces us to reconsider our understanding of both the nature and incidence of neural stem cells in the adult brain and challenges our conception of the supposed rarity of adult neural progenitor and stem cells. In doing so, they point to an abundant and widespread source of cells, which may be used both as a target for pharmacological induction and as a cell type appropriate for therapeutic engraftment to the diseased adult brain.

THERAPEUTIC POTENTIAL OF HUMAN OLIGODENDROCYTE PROGENITOR CELLS

The Natural History of Remyelination in the Adult CNS

The existence of active remyelination in the adult human brain has been mainly derived from observations of MS lesions. However, it has been unclear whether that remyelination has been the result of local expansion of parenchymal OPCs or of the recruitment of distant OPCs to sites of acute demyelination. Moreover, the source and in resting phenotype of the

remyelinating cells has been unclear. To address these questions, Gensert and Goldman (1997) used a combination of retroviral labeling and lysolecithin-induced demyelination to show that normally cycling cells of the adult rodent WM can differentiate as myelinating oligodendrocytes (Gensert and Goldman, 1997). Interestingly, before the endogenous OPCs participated in remyelination, they proliferated locally. Similarly, mice infected with a demyelinating murine hepatitis virus exhibited almost a 14-fold increase in PDGF-αR^+ OPCs in the lesion bed (Redwine and Armstrong, 1998). Other studies using rats with EAE or ethidium bromide lesions have shown that after remyelination, OPC numbers were stable (Levine and Reynolds, 1999). This in turn suggested that OPCs can undergo asymmetric division to replicate themselves while generating a differentiating oligodendrocyte.

There appears to be limited survival of OPCs in demyelinated lesions; as a result, most remyelination may be accomplished by unaffected OPCs recruited from the lesion surround. Carroll *et al.* have shown that OPCs in regions adjacent to immunolytic lesions first respond by dividing, followed by their migration into the lesion, and ultimate myelinogenesis (Carroll *et al.*, 1998). Similar observations were made in the demyelinated adult spinal cord, where the population of $NG2^+$ cells expanded significantly in areas adjacent to demyelinating lesions. In this case though, the proliferating pool appeared unable to sustain its self-renewal, as $NG2^+$ cells were depleted following remyelination (Keirstead *et al.*, 1998). Using X-irradiation, Chari and Blakemore (2002) reported that locally recruited $NG2^+$ and PDGF-α R^+ OPCs can repopulate depleted areas over distances of approximately 0.5 mm per week in the first month. No secondary progenitor loss was observed in those surround regions from which progenitor cells were recruited, indicating dynamic replacement of the emigrants (Chari and Blakemore, 2002). However, the question of how far the progenitor population can migrate in intact tissue remains debatable, an issue of particular concern for remyelination strategies involving transplantation (Franklin and Blakemore, 1997). Complicating matters further, recent studies have reported an age-related decrease both in recruitment of OPCs and in their subsequent differentiation (Sim *et al.*, 2002).

Candidate Cellular Vectors for Experimental Remyelination via Progenitor Implantation

Progenitor cells capable of local cell genesis therefore persist throughout the subcortical white matter of the adult brain, where they might constitute a potential substrate for cellular replacement and local repair. However, several criteria must be considered when evaluated the transplantation potential of any progenitors. These include the ability of transplanted cells to survive in the host environment, to migrate accurately to the target lesion or tissue type, to generate myelin, to ensheath host axons, and to achieve a degree of myelination capable of functional reconstitution. To assess the myelinogenic potential of transplanted cells, a variety of cell types including multipotential stem cells and OPCs, derived from both animals and humans, have been tested in both developmentally dysmyelinated and experimentally demyelinated models of myelin loss.

Neural Stem Cells and Progenitors from the Fetal Brain

Human fetal brain cells have been found to have robust myelinogenic capacity in the *shiverer* mice (Gansmuller *et al.*, 1986; Gumpel *et al.*, 1987, 1989). Cells isolated from the rodent or human fetal forebrains at various gestational ages, and expanded *in vitro* under a variety of serum-free, factor-supplemented conditions, have been used as sources of transplantable cells (Ader *et al.*, 2001; Brustle *et al.*, 1998; Carpenter *et al.*, 1999; Fricker *et al.*, 1999; Hammang *et al.*, 1997). However, there are potential risks to prolonged *in vitro* expansion, since the cells are not only exposed to exogenous mitogens, but also to autocrine factors in artificially high concentration, and to paracrine agents produced by the neurons and glia present within the initially mixed cultures. As a result, propagated stem or progenitor cells may not retain or reflect the lineage potential or differentiation competence of the native progenitor cells from which they derived. Two recent studies have highlighted the effects of *in vitro* expansion of cells prior to transplant. Buchet *et al.* observed that freshly isolated cells

proliferated longer and gave rise to very extended grafts before differentiating into neurons and glia while cells that were expanded prior to transplant showed poor proliferation and quick differentiated capacity (Buchet *et al.*, 2002). In contrast, Englund *et al.* found that after 9 weeks of expansion, human fetal brain cells lost the capacity to differentiate and remained as undifferentiated progenitors when transplanted into adult recipients (Englund *et al.*, 2002). To circumvent the issue of paracrine effects on defined stem cells in mixed culture, several groups have developed methods of directly isolating neural stem cells from tissue, thereby preventing their *in vitro* exposure to differentiated cell products during either isolation or expansion (Keyoung *et al.*, 2001; Uchida *et al.*, 2000).

Neural Stem Cells and Progenitors from Adult Brain

Several studies describe the use of neural stem cells derived primarily from the adult rat and human VZ, and then propagated as neurospheres, as a potential source of myelinogenic cells (Akiyama *et al.*, 2001; Kukekov *et al.*, 1999; Zhang *et al.*, 1999). As described earlier, the adult human white matter harbors an abudance of oligodendrocyte progenitors. By virtue of their abudance, these progenitors represent a potential cellular substrate for therapeutic transplantation. Nonetheless, only a few studies, constrained by the lack of any reliable method to isolate these cells, have attempted to assess the myelinogenic capacity of OPCs derived from the adult human white matter. In one such study (Targett *et al.*, 1996), a crude cell preparation derived from adult human white matter was transplanted into the ethidium bromide-lesioned and radiosensitized, X-irradiated adult rat spinal cord. The transplanted oligodendrocytes survived in the demyelinated zone, associated with denuded host axons, and expressed myelin proteins. But the transplanted cells did not migrate or divide, nor was any myelination noted. The failure of these implanted oligodendrocytes to myelinate was attributed to the diminished regenerative potential of post-mitotic oligodendrocytes, and the lack of a permissive environment for remyelination within the rat lesion bed (Targett *et al.*, 1996).

Propagated Oligospheres

Though neural stem cells have myelinogenic capacity, they also have the inherent capacity to generate neurons and astrocytes. The co-generation of astrocytes may not necessarily be deleterious, given their roles in both OPC proliferation and myelination (Blakemore, 1992; Franklin *et al.*, 1991). However, the co-generation of neurons may be undesirable, given the potential generation of ectopic neuronal foci, which might conceivably act as epileptogenic foci. Thus, priming neural stem cells or OPCs toward oligodendrocytic differentiation prior to implant might be necessary to ensure the quantities and phenotypic homogeneity of oligodendrocyte progenitor cells that will be needed for clinical implantation. One approach to this goal has been the expansion of neural stem cells as neurospheres in the presence of oligodendrocyte-inducing agents. For instance, when rat cerebellum-derived neurospheres were propagated in the presence of conditioned medium from the neuroblastoma B104 line (B104/CM), oligodendrocytes were preferentially generated. The resultant "oligospheres" were capable of being exponentially expanded through several passages without phenotypic degradation and exhibited robust myelination on transplantation into the *shiverer* mice brain (Avellana-Adalid *et al.*, 1996). Since then, several groups have used a similar strategy to generate oligospheres from neural precursor cells of the mouse, rat, and canine forebrains (Vitry *et al.*, 1999; Zhang *et al.*, 1998). Smith and Blakemore the compared the remyelinating capacity of cells isolated from porcine SVZ within hours after dissociation, to that exhibited by matched cells after growth in B104/CM as oligospheres. Whereas the freshly isolated SVZ cells remained undifferentiated after xenograft, those expanded in B104/CM effected significant remyelination of demyelinated axons *in vivo* (Smith and Blakemore, 2000).

Human OPCs Integrate When Grafted to Demyelinated Foci of the Adult Rat Brain

The remyelinating potential of adult human white matter-derived progenitors has been recently shown in lysolecithin-induced demyelinating lesions of adult rat corpus callosum

(Windrem *et al.*, 2002). In this study, A2B5 expression by P/CNP2:hGP-defined OPCs (Roy *et al.*, 1999) provided the rationale for immunomagnetically selecting OPCs on the basis of A2B5 expression. Like P/CNP2:hGFP$^+$ cells, A2B5-sorted cells generated largely oligodendrocytes when raised at high density in the presence of serum. In addition, immunomagnetic selection allowed their higher-yield acquisition, without the losses in viability and number associated with FACS separation. As a result, A2B5-antibody based immunomagnetic sorting increased the yield of extractable OPCs by over 5-fold. These A2B5-sorted white matter progenitors were transplanted into cyclosporine-immunosuppressed adult rats, 3 days after lysolecithin lesions. As previously described (Gensert and Goldman, 1997), these lesions yielded a discrete region of transcallosal demyelination, with mild local reactive astrocytosis within the demyelinated focus, and intact vasculature. When A2B5-sorted human OPCs were injected into these lesions, they migrated widely and rapidly; within 7 days of implantation, the cells had readily traversed the midline to infiltrate the furthest reaches of the demyelinated lesion beds, which often extended over 8 mm in breadth. The migration rate of the cells was hence at least 1 mm/day, or 50 μm per hour, within the lesion borders (Fig. 10.5). The engrafted adult A2B5-sorted progenitors differentiated rapidly, expressing CNP within 2 weeks and MBP within 3 weeks of implantation. These OPC-derived oligodendrocytes projected MBP$^+$ lamellopodia and were associated with a branched array of myelinating fibers, indicating the initiation of progenitor-associated myelinogenesis. Of note, many transplanted progenitor derived astrocytes were also observed in the lesions. With cyclosporine immunosuppression, the cells could survive at least 2 months in lysolecithin-demyelinated recipients. These findings suggested that the introduction of highly enriched preparations of progenitor cells derived from the adult human white matter might permit local remyelination.

Migratory Characteristics of human OPCs

Adult human-derived OPCs engrafted into demyelinated brain remained restricted to regions of demyelination; they rarely extended into normal surrounding myelin (Fig. 10.5). Even the few cells that were typically noted to have infiltrated normal myelin appeared to have migrated along the extraluminal surfaces of penetrating blood vessels. Yet when lentiviral-GFP tagged A2B5-sorted progenitor cell pools from adult human white matter were implanted into intact subcortex of adult rats, the transplanted cells remained localized to the implant site and continued to be so even after 3 months (Windrem *et al.*, 2002). These observations suggest strongly that normal adult white matter is non-permissive for the migration of adult-derived WMPCs, as has been observed in other studies (Iwashita *et al.*, 2000). This restriction on migration may be similar at the molecular level to that observed toward axons, whose extension through normal white matter is suppressed by their expression of Nogo receptor, by which they respond to myelin-associated Nogo and MAG (myelin-associated glycoprotein) with repulsion and/or cessation of further advance (Grandpre and Strittmatter, 2001). That being said, the operative white matter signals that restrict progenitor cell migration have yet to be identified. Whatever its mechanism, normal myelin clearly retains cues sufficient to tonically impede WMPC infiltration; accordingly, demyelination appears to remove those cues, allowing the active invasion and dispersion of OPCs throughout regions of acute myelin loss. The characterization of the ligands providing these repulsive cues, and of their anticipated progenitor cell receptors, will likely constitute an important avenue of future study.

Myelin Construction by Perinatal Transplant-Based Therapy

Several models of congenital dysmyelination have been used to assess the myelinogenic potential of animal and human-derived progenitors. The myelinogenic potential of implanted fetal human brain cells was first noted in the shiverer mouse (Gumpel *et al.*, 1987; Lachapelle *et al.*, 1983). The myelinogenic potential of different, stage-defined phenotypes of oligodendrocyte progenitors, extracted so as to sample the engraftment efficacy of different stages of progenitor progression, have also been compared in shiverer mice. Using rat donor tissue, Warrington and Pfeiffer established that the A2B5-defined oligo-

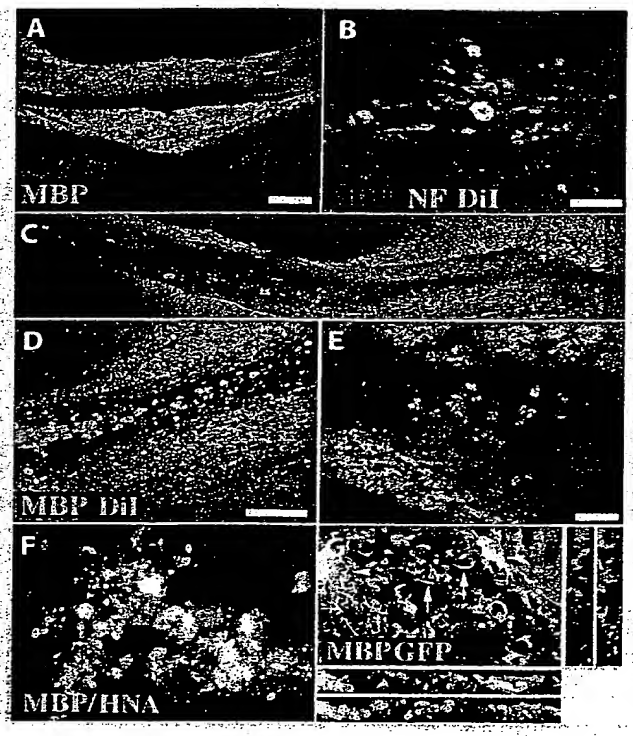

FIGURE 10.5
Implanted white matter progenitors migrated widely throughout the demyelinated callosum. Sorted adult human white matter progenitors were transplanted into lysolecithin-induced demyelinated lesions in the corpus callosa of adult rats. (A) Lysolecithin infusion (1 µl of 2% lysolecithin-V, delivered into the corpus callosum) yielded demyelinated plaques in the target white matter. In part A, the lesion is visible as a discoid region of myelin basic protein (MBP)-immunonegativity, surrounded by the otherwise MBP^+ callosum (green). (B) Though denuded of myelin (MBP, blue), neurofilament$^+$ axons (green) initially survived lysolecithin demyelination, 1 week after callosal lesion. The implanted progenitors (orange) have just immigrated to the lesion bed. Axonal spheroids were frequent within the lysolecithin-lesion bed, indicating some degree of early injury and transection. The ability of implanted progenitors to effect repair is limited by the viability and integrity of the axonal cohort that one wishes to myelinate. (C) This low-power montage demonstrates the rapidity of long-distance migration by xenografted adult human white matter progenitors. These DiI-labeled human progenitor cells (red) were visualized 1 week after their implantation, by which point the cells extend throughout the demyelinated lesion, defined by its loss of myelin basic protein (MBP)-immunoreactivity (green). The lesion was induced 3 days before 10^5 sorted, DiI-tagged (red) human progenitors were delivered in 2 µl. Within a week of implantation into this demyelinated callosum, the cells had traversed the midline. (D) A higher magnification image showing that the transplanted cells migrated throughout the demyelinated plaque, but not beyond its borders, except for occasional migrants that followed the parenchymal surfaces of blood vessels (arrow). The restriction of migration to demyelinated regions suggests that normal myelin impedes the migration of these cells. (E) Human white matter progenitor cells, identified as human nuclear antigen$^+$ (HNA; green), occupied the MBP (green)-deficient lysolecithin lesion, and expressed oligodendrocytic CNP (red) by 15 days after implantation. (F) A cluster of HNA^+ human cells (green) associated with a plethora of donor-derived MBP^+, myelinating oligodendrocytic lamellopodia (red). (G) Lentiviral GFP-tagged human (green) MBP^+ (red) oligodendrocytes in the lesion bed of a lysolecithin-injected rat callosum, 8 weeks after cell implantation. Besides the MBP^+ cells (arrows), other human progenitor-derived cells were also present, which did not express MBP and which instead manifested astrocytic morphologies (arrowheads). Immunolabeling adjacent sections for human GFAP (red) revealed that many of GFP-tagged human progenitors had also given rise to astrocytes. From Windrem *et al.* (2002). Scale bars: A, 200 µm; B, 20 µm; D, 100 µm; E, 30 µm.

dendrocyte progenitor phenotype was more efficient at migration and myelinogenesis in neonatal shiverers than the more mature O4-defined oligodendrocyte (Warrington *et al.*, 1993). Yandava *et al.* similarly achieved myelination within the shiverer brain, using the C17.2 line of transformed murine cerebellar progenitor cells, which act as neural stem cells after v-myc immortalization (Yandava *et al.*, 1999).

Similarly, fetal oligodendrocytes transplanted to the *md* rat remyelinated significant portions of the postnatal spinal cord (Archer *et al.*, 1994). Moreover, analogous studies in the *shaking* pup showed that fetal oligodendrocytes were able to engraft widespread regions of the *shaking* CNS, with graft survival of over 6 months. Although neonatal recipients fared best, adult recipients also exhibited graft oligodendrocyte survival and stable myelination (Archer *et al.*, 1997). Duncan and colleagues then demonstrated that oligosphere-derived cells raised from the neonatal rodent subventricular zone could engraft another dysmyelinated mutant, the myelin-deficient rat, upon perinatal intraventricular administration (Learish *et al.*, 1999). The success of these approaches led then to the seminal work of Mitome and colleagues, who used EGF responsive primary neural progenitor cells, in tandem with a combination of ventricular and cisternal transplant, to achieve the widespread myelination of the shiverer brain (Mitome *et al.*, 2001).

Human OPCs Can Myelinate Congenitally Dysmyelinated Brain

On the basis of these studies, Windrem *et al.* investigated whether highly enriched populations of human progenitor cells, directly isolated from the brain, might be used for cell-based therapy of congenital dysmyelination. Specifically, this study postulated that the efficiency of myelination might be improved by using purified OPCs, derived via selection so as to exclude astrocytes, microglia, and vascular derivatives from the implanted pool. It further postulated that such purified human OPCs, both adult-derived and taken from the fetal brain during its period of maximal oligoneogenesis, would be sufficiently migratory and myelinogenic to mediate the widespread myelination of a perinatal host. To this end, A2B5-based FACS was used in conjunction with PSA-NCAM-dependent immunodepletion of neuronal derivatives, to prepare highly enriched dissociates of human OPCs, of both fetal and adult derivation. Both classes of human oligodendrocyte progenitor cells proved capable of widespread and high-efficiency myelination of the shiverer brain after perinatal xenograft. Indeed, the cells migrated so widely as to effect myelination throughout the recipient brains (Fig. 10.6, unpublished data). The cells infiltrated widely throughout the presumptive white matter, ensheathed resident murine axons, and formed antigenically and ultrastructurally compact myelin. After implantation, the cells slowed their mitotic expansion with time and generated neither undesired phenotypes nor parenchymal aggregates. In this initial study, despite histologically extensive myelination in these animals, no change in the behavioral phenotype of the shi/shi recipients or any improvement in their neurological phenotype was evident. Nonetheless, the geographic extent of forebrain and diencephalic MBP expression evidenced by these animals, who received but a single perinatal intraventricular cell injection, suggested that combined cisternal and intraventricular delivery of donor progenitors might achieve remyelination throughout the rostral neuraxis, potentially spanning the entire brain.

Besides demonstrating the myelinogenic capacity of the transplanted cells, studies in the dysmyelinated animal models indirectly indicate that congenital dysmyelination, even more so than adult demyelination, may be an appropriate target for CNS progenitor cell-based therapy. In particular, these studies affirmed that the neonatal brain environment may be especially amenable to therapeutic remyelination. It is conducive to widespread migration and may continue to provide the instructive developmental cues necessary for region-specific differentiation.

Fetal and Adult OPCs Differ

Despite the use of both fetal and adult-derived OPCs in experimental therapeutic models, no head-to-head comparison of the two phenotypes had ever been performed from

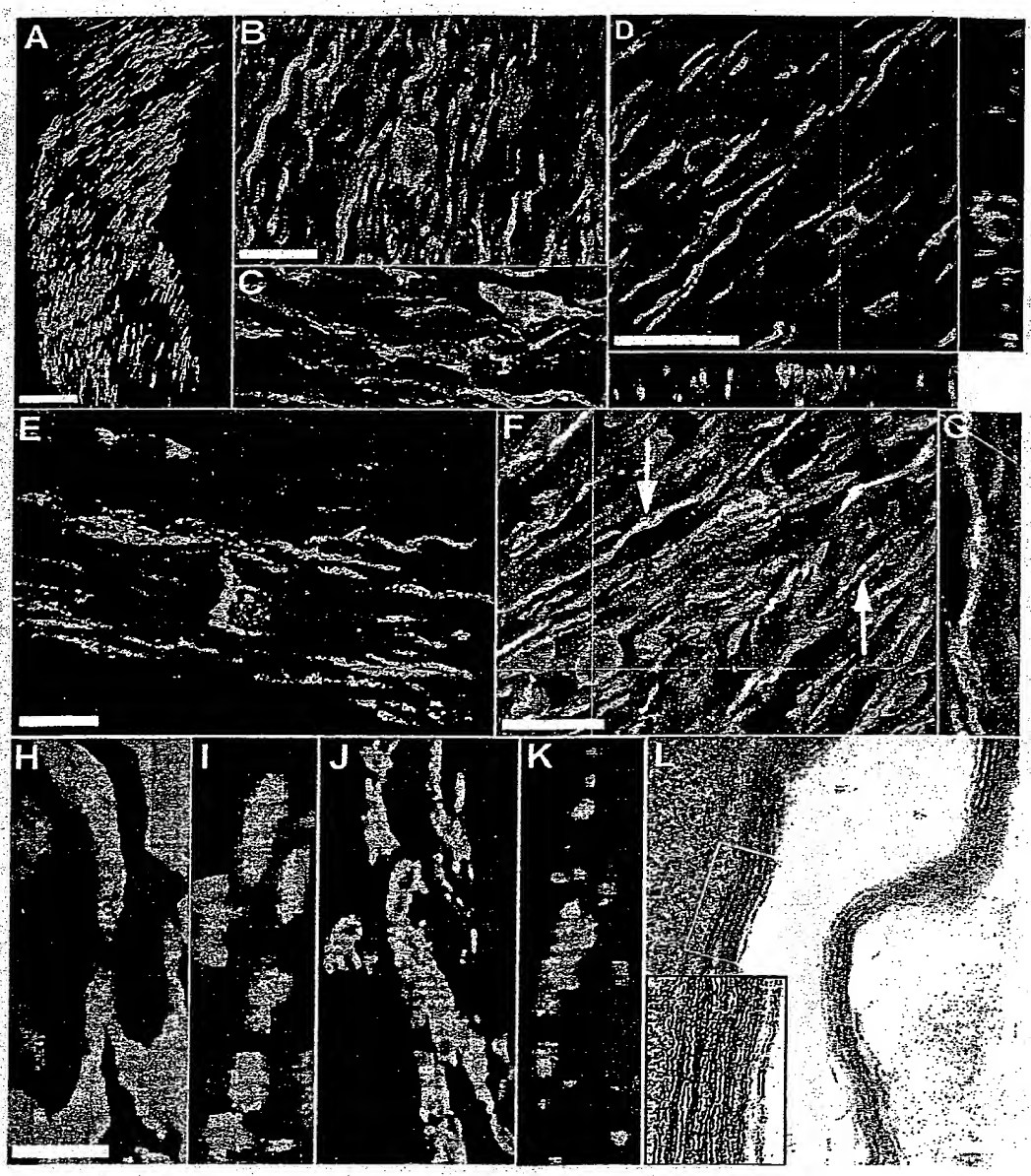

FIGURE 10.6

Myelin basic protein (MBP) was widely expressed by human fetal OPCs implanted into neonatal homozygote shiverer mice. (A) This low-power view of the recipient fimbria shows abundant fiber-associated MBP expression (*green*). 3 months after perinatal engraftment (MBP, *green*) Since shiverer homozygotes do not express immunoreactive MBP, all such signal must derive from donor progenitor cells. (B-D) Donor fetal OPCs, additionally validated as such by human nuclear antigen (hNA, *red*), differentiated to express CNP protein (*green* in B) and MBP (*green* in C-D). In the 0.5 μm confocal optical section of D, MBP (*green*) is noted to surround the donor human nucleus (*red*), as viewed in orthogonal planes. (E). A single donor-derived MBP^+ oligodendrocyte that has matured, 3 months after engraftment, to associate with multiple recipient axons. (F) An 0.2 μm optical section through a recipient corpus callosum shows engrafted human OPCs (hNA, *blue*) expressing MBP (*red*), and surrounding native axons (neurofilament, *green*). Arrows indicate examples of ensheathed axons, a higher magnification of which is shown in (G). Human OPCs enwrap native axons and reorganize the paranodal region to permit nodal formation. Caspr protein, an axonal paranodal marker, is expressed on unmyelinated axons between myelinated segments of axon, without invading the nodal region. (H-K) Optical sections through engrafted shiverer corpus callosum, showing donor-derived MBP (*green*) and native axonal Caspr protein in red, indicating that donor OPCs develop not only myelin production and architecture, but permit nodes of Ranvier to form (anti-Caspr antibodies generously provided by Dr. M. Rasband). (L) Electron microscopy confirmed that donor-derived oligodendrocytes developed compact myelin, I that myelin produced by engrafted fetal human OPCs wrapped native axons to form compact sheathes with major dense lines (*inset*). Scale bars: A, 200μm; B-F, 20μm; H-K, 5 μm; L, 1 μm.

analogously acquired and maintained cells implanted into the same models at the same time. As a result, it was unclear if fetal-derived OPCs differed from their counterparts derived from the adult human brain, with respect to either their migration competence, myelinogenic capacity, or the time courses thereof. To assess the relative advantages and disadvantages as therapeutic vectors of these two stage-defined OPC phenotypes, newborn shiverer mice were implanted with either fetal or adult-derived OPCs, each isolated via A2B5-directed immunomagnetic sorting (IMS). The implanted neonatal mice were allowed to survive for 1 to 3 months, and their brains then sectioned and stained for MBP, GFAP, and anti-human nuclear antigen. By this means, it was determined that fetal and adult-derived human OPCs differed substantially in their respective time courses and efficacy of myelinogenesis upon xenograft. Adult OPCs myelinated shiverer brain more rapidly than their fetal counterparts, achieving widespread and dense MBP expression by 4 weeks after xenograft. In contrast, substantial MBP expression by fetal OPCs was generally not observed until 12 weeks post-implant (Windrem *et al.*, unpublished data).

Besides myelinating more quickly than fetal OPCs, adult OPCs were found to give rise to myelinogenic oligodendrocytes in much higher relative proportions, and with much less astrocytic co-generation, than did fetal-derived OPCs. When assessed at the midline of the recipient corpus callosum, just over 10% of fetal hNA-defined OPCs expressed MBP at 12 weeks, while virtually none had done so at 4 weeks. In contrast, almost 40% of adult OPCs expressed MBP by 4 weeks after xenograft into matched recipients. Thus, engrafted adult OPCs were at least four times more likely to mature as oligodendrocytes and develop myelin than their fetal counterparts. As another cardinal difference between fetal and adult OPCs, adult OPCs largely remained restricted to the host white matter, within which they generated almost entirely MBP^+ oligodendrocytes. In contrast, fetal OPCs migrated into both gray and white matter, generating both astrocytes and oligodendrocytes in a context-dependent manner.

Thus, both fetal and adult-derived OPCs were competent to remyelinate murine axons, but each had distinct advantages and disadvantages as potential vectors for cell therapy: Whereas fetal OPCs were highly migratory, they myelinated slowly and inefficiently. In contrast, adult-derived OPCs migrated over lesser distances, but they myelinated more rapidly and in higher proportions than their fetal counterparts. Together, these studies argued that while both fetal and adult human OPCs might provide effective cellular substrates for remyelination, the choice of cellular source must be dictated not only by the availability of donor material, but also by the specific biology of the disease target.

A Caveat: Some Implanted Progenitors May Remain Undifferentiated

A corollary of the multipotential nature of white matter progenitor cells is that when transplanted as nominally oligodendrocytic precursors, these cells might encounter local signals that instruct their maturation along alternative lineages. As a result, we need to be concerned about the possibility of their differentiation into undesired or functionally heterotopic phenotypes. This possibility is of further concern given the persistence of many implanted progenitors as undifferentiated cells; these may remain able to respond to signals in the host tissue environment, not only at the time of implantation, but also long thereafter. As such, these cells might comprise a reservoir of implanted precursors, from which desired phenotypes might be later recruited upon injury or insult. On the other hand, they might just as well constitute potential sources of undesired cell types that might be ectopically generated and recruited in the tissue environment of an acutely injured focus. Such local production of undesired phenotypes might introduce not only inefficiency to transplant-based treatment strategies, but also frank danger. For instance, the production of neurons in a white matter lesion could generate an epileptogenic focus, just as the production of astrocytes in a more typically oligodendrocytic region might disrupt local ionic gradients and hence axonal transmission. These and many other untoward processes of heterotopic phenotypic maturation could more than offset whatever benefits might be gleaned from a therapeutic cell implant. As a result, it may prove advisable to initiate the phenotypic differentiation of these cells *in vitro*, prior to implantation, so as to limit

the range of phenotypic choices available to the isolated progenitors to those appropriate for the intended region and disease target. Time will tell whether the possibility of heterotopic misdifferentiation will mandate such *in vitro* priming steps.

EXPERIMENTAL IMPLANTATION OF NON-CNS PROGENITOR CELL TYPES

A wide range of other potentially myelinogenic cell types have also been implanted into experimental models of de- and dysmyelination, with varying degrees of success.

Schwann Cells

Schwann cells, the myelinating cells of the peripheral nervous system, have been considered as an attractive alternative to oligodendrocyte precursors for experimental transplantation. Schwann cells from several sources, including humans (Kohama *et al.*, 2001), have been implanted in dysmyelinated *shiverer* mice (Baron-Van Evercooren *et al.*, 1992), MD rats, and *shaking* pups (Duncan and Hoffman, 1997). They have also been transplanted into lysolecithin (Baron-Van Evercooren *et al.*, 1993; Duncan *et al.*, 1981) and EB-X (Blakemore and Crang, 1985) demyelinated lesions in the brain and spinal cord. In all these systems, they have demonstrated varying degrees of myelination (Franklin and Barnett, 1997) with the myelin produced by these cells being of the PNS-variety as specified by the expression of P0. In some cases, functional reconstitution of saltatory conduction has also been shown (Felts and Smith, 1992; Honmou *et al.*, 1996; Kohama *et al.*, 2001). In addition, Schwann cells have been reported to improve axonal regeneration, which might be of importance in MS where axonal loss is a major part of the lesion pathology. Considering the relative ease of expanding human Schwann cells in culture (Rutkowski *et al.*, 1995), it has been suggested that they might be appropriate cellular vectors for autologous transplants. Indeed, they have the added advantage of producing non-CNS myelin, which may be refractory to the immunological destruction in diseases like MS. However, like central oligodendrocyte progenitors, the migratory capacity of these cells is unclear. Some studies indicate that Schwann cells migrate satisfactorily over large distances to specific target sites (Franklin and Barnett, 1997), while others indicate that they are unable to migrate through normal white matter (Iwashita *et al.*, 2000). In addition, Schwann cells seem to have a complex relationship with central astrocytes. After transplantation, Schwann cells are mainly found in areas devoid of astrocytes (Baron-van Evercooren *et al.*, 1992; Blakemore and Patterson, 1975), and, moreover, they are excluded as astrocyte numbers increase with time (Shields *et al.*, 2000).

Olfactory Ensheathing Cells (OEC)

In nature, OEC's ensheath small diameter axons of the peripheral olfactory epithelium neurons that project through the olfactory nerve into the olfactory bulb of the CNS. Unlike Schwann cells, these cells do not normally produce myelin. However, OECs from both animal (Franklin *et al.*, 1996; Imaizumi *et al.*, 1998) and human sources (Barnett *et al.*, 2000; Kato *et al.*, 2000) show remyelination with a peripheral pattern of myelin expression upon transplantation to demyelinated spinal cords. In some studies, a functional restoration of conduction has also been demonstrated (Imaizumi *et al.*, 2000). OECs may have an advantage over Schwann cells, as they co-exist naturally with astrocytes in the olfactory bulb (Lakatos *et al.*, 2000). Perhaps as a result, their interaction with astrocytes in not restrictive (Franklin and Barnett, 2000) in fact, they have been reported to support axonal regeneration through the astrocytic environment of a transected spinal cord (Ramon-Cueto *et al.*, 1998). Nonetheless, their restoration of central axonal conduction remains inconclusive, as is the long-term fate of their remyelinated units. Whether these cells are capable of the contact-dependent and humoral support of neuronal function

normally exercised by central oligodendrocytes, or conversely, whether they are in turn supported by the axons with which they interact (Fernandez *et al.*, 2000; Vartanian *et al.*, 1997), similarly remain unknown.

Embryonic Stem Cells

Myelination by *in vitro* conditioned mouse embryonic stem cells has been reported in both hypomyelinated MD rat E-17 fetuses and *shiverer* newborns, as well as in adult lysolecithin demyelinated lesions in adult rats (Brustle *et al.*, 1999; Liu *et al.*, 2000). More recent reports describe transplanted human ES cells sequentially cultured to induce neural stem cells capable of generating oligodendrocytes in a region-specific manner (Reubinoff *et al.*, 2001). Though ES cells might represent a readily cultivable source of OPCs, the use of these cells is still limited by our inability to fully instruct all cells in the undifferentiated population to the desired phenotype. Of greater concern is the persistent uncommitted progenitors within the implanted population, which may retain the latent capacity for undifferentiated expansion and possibly tumorigenicity.

Mesenchymal and Marrow-Derived Stem Cells

In addition to ES cells, mesenchymal and marrow-derived stem cells have been in focus as a source of neurally specified cells. Some controversial studies indicate that these cells may be capable of trans- or ectopic differentiation to neuroectodermal lineage (Mezey *et al.*, 2000; Sanchez-Ramos *et al.*, 2000). Of particular concern has been the lack of clear clonal evidence of neural specification as well as recent reports indicting that cell fusion may explain some of observations of trans-differentiation (Terada *et al.*, 2002; Ying *et al.*, 2002). Nonetheless, a recent study, in which mouse bone marrow stromal cells were grafted into EB-X demyelinated spinal cord lesions, reported not only donor-cell derived histological remyelination, but also an improvement in conduction velocity (Akiyama *et al.*, 2002). This work remains to be replicated by other groups. Should this study prove verifiable, its approach may open new avenues of stromal cell-based remyelination therapy.

DISEASE TARGETS FOR PROGENITOR-BASED THERAPEUTIC MYELINATION

Congenital Dysmyelination

Congenital diseases of myelination, such as periventricular leukomalacia (PVL), which may serve as an anatomic form fruste for the later development of cerebral palsy (Grow and Barks, 2002; Rezaie and Dean, 2002; Volpe, 2001) and the hereditary leukodystrophies and storage diseases, such as Krabbe's and Tay Sachs disease, are leading causes of infant morbidity and mortality (reviewed by Schiffmann and Boespflug-Tanguy, 2001; Berger *et al.*, 2001). As such, these may constitute feasible and attractive targets for therapeutic remyelination (Tate *et al.*, 2001).

Periventricular leukomalacia PVL describes a lesion of the periventricular white matter, associated with a failure in early myelination of the cerebral hemispheres. PVL appears to be a pathological concomitant to perinatal hypoxic-ischemic insult and may result from germinal matrix hemorrhage, sustained hypoxia, and excitotoxic injury, and most likely from combinations of these insults. PVL predicts the development of cerebral palsy in most cases (Volpe, 2001). Experimental models of hypoxic-ischemia in neonatal rats (Back *et al.*, 2002; Levison *et al.*, 2001) as well as studies of pediatric autopsies (Back *et al.*, 2001) have suggested that the late oligodendrocyte progenitors of the forebrain SVZ comprise the predominant cell population lost in perinatal ischemic injury. This is in accord with our understanding of the natural history of oligodendrogliogenesis in humans (Grever *et al.*, 1999; Rakic and Zecevic, 2003; Zhang *et al.*, 2000), the developmental window for which corresponds to the period of ischemic vulnerability of the periventricular white matter.

Congenital leukodystrophies include an ever-expanding group of inherited diseases of myelin synthesis and metabolism. Although a diverse group, these may roughly be divided into lysosomal storage diseases, such as Krabbe's globoid cell leukodystrophy (Wenger *et al.*, 2000) and Tay Sachs diseases (Gravel *et al.*, 1991); disorders of myelin synthesis, such as Pelizaeus-Merbacher disease (PMD) (Koeppen and Robitaille, 2002); and metabolic deficiencies leading to toxic demyelination, such as Canavan's disease (Matalon and Michals-Matalon, 2000). Each of these disease categories is attended by extensive white matter involvement and clinical leukoencephalopathy, typically leading to severe neurological disability and death. As a group, the clinical leukodystrophies represent especially attractive targets for progenitor cell-based therapy, since the restoration of healthy oligodendrocytes in early perinatal development may be sufficient to permit myelination and hence to slow or prevent the development of the disease phenotype. In addition, effective murine models of these diseases are available (Werner *et al.*, 1998). Inherited diseases of the PLP and MBP genes are modeled by twitcher (Mikoshiba *et al.*, 1985; Yoshimura *et al.*, 1989) and shiverer mice (Roach *et al.*, 1985), respectively. In addition, mutations of hexoseaminidase-B, modeling Sandhoff's and Tay-Sachs diseases (Kolter and Sandhoff, 1998), and aspartoacylase, mimicking Canavan's disease (Matalon *et al.*, 2000), have been similarly employed. The availability of such genetically precise models of the childhood leukodystrophies is already greatly accelerating the process of developing experimental treatment strategies for these disorders.

Acquired Demyelination

In adults, the diseases of acquired demyelination include later-onset leukodystrophies, such as metachromatic leukodystrophy and adrenoleukodystrophy, as well as vascular, inflammatory, and nutritional demyelinating syndromes (Baumann and Turpin, 2000; Berger *et al.*, 2001; Desmond, 2002; Dichgans, 2002). The vascular demyelinations include hypertensive and diabetic leukoencephalopathies, which may both be due to chronic oligodendrocytic ischemic hypoxia (Dewar *et al.*, 1999; Leys *et al.*, 1999). Subcortical strokes, particularly those within the distributions of the forebrain lenticulostriate and thalamogeniculate arterial territories, are also prominent causes of vascular demyelination (Dichgans, 2002). The inflammatory demyelinations include multiple sclerosis, transverse myelitis (Kerr and Ayetey, 2002), optic neuritis (Cree *et al.*, 2002; Eggenberger, 2001), and less commonly Schilder's leukoencephalitis (Kotil *et al.*, 2002), as well as postvaccinial (An *et al.*, 2002; Konstantinou *et al.*, 2001) and postinfectious leukoencephalitis (Kleinschmidt-DeMasters and Gilden, 2001; Rust, 2000). All of these syndromes of acquired demyelination are potential targets of therapeutic remyelination. Yet most attempts at cell-based remyelination in experimental animals have been made using acute chemical demyelinating insults, such as lysolecithin. In contrast to the availability of effective animal models of congenital dysmyelination, the study of acquired demyelination has suffered from its lack of biologically appropriate, clinically reflective animal models. As a result, few adequate studies of cell-based remyelination of acquired, adult demyelinating lesions have been reported using any cellular vector. Those studies that have reported oligodendrocytic maturation and myelination by implanted oligodendrocyte progenitors have typically failed to demonstrate substantial axonal ensheathment, though this has likely reflected the loss of competent axons in these models, rather than any insufficiency on the part of the implanted progenitor cells. Indeed, the etiological complexity and manifold sequelae of demyelination in the adult brain argues against easy therapeutic intervention. As such, until improved models of acquired demyelinating disease are available, progress in cell-based therapy of adult demyelinating diseases will be necessarily slow. In contrast, the arguably simpler etiologies of congenital dysmyelination, their frequent lack of association with underlying systemic disease, and the persistent structural plasticity of the perinatal brain, together with the many effective animal models for congenital dysmyelination, collaborate to make these diseases attractive targets for near-term intervention, both experimentally and clinically. Indeed, we may reasonably except the congenital leukodystrophies to be especially promising targets for cell-based therapeutic remyelination.

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The O-2A^{adult} progenitor cell: a glial stem cell of the adult central nervous system

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Systematic comparison of the properties of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from optic nerves of perinatal and adult rats has revealed that these two populations differ in many fundamental properties. In particular, O-2A^{perinatal} progenitor cells are rapidly dividing cells capable of generating large numbers of oligodendrocytes over a relatively short time span. Oligodendrocyte differentiation generally occurs synchronously in all members of a clone, thus leading to elimination of that clone from the pool of dividing cells. However, some O-2A^{perinatal} progenitors are also capable of giving rise to O-2A^{adult} progenitors. These latter cells express many of the characteristics of stem cells of adult animals, including the capacity to undergo asymmetric division and differentiation. We suggest that precursors which function during early development give rise to terminally differentiated end-stage cells and to a second generation of precursors with properties more appropriate for later developmental stages. It is this second generation of precursors which express the properties of stem cells in adult animals, and we therefore further suggest that our work offers novel insights into the possible developmental origin of stem cells.

Key words: progenitor / astrocyte / *perinatal* / *adult*

IN THE LIGHT OF the considerable physiological differences between development and maturity, it has seemed likely that precursor cells which contribute to the early generation of a tissue and the precursors involved in replacement of cells in older animals might differ in their properties. The explosive growth of embryogenesis would be inappropriate in most adult tissues, and processes must exist for slowing down this rapid generation of cells. However, precursor populations cannot be entirely eliminated, as there is a need in adult tissues for the maintenance of a population of precursor cells which would have the capacity to contribute to tissue repair. It is these cells which include the stem cells of adult tissue.

For at least several different cellular lineages, substantial differences have indeed been demonstrated between the precursor cells present during development and in the adult animal. For example, there are fundamental differences between embryonic myoblasts and adult muscle satellite cells,[1,2] and between fetal and adult cells of both the haematopoietic and sympathoadrenal lineages.[3,4] In addition, although optic nerves of both perinatal and adult rats contain progenitor cells which can be induced to differentiate *in vitro* into either oligodendrocytes or type-2 astrocytes,[5-10] the oligodendrocyte-type-2 astrocyte (O-2A) progenitors isolated from optic nerves of adult rats differ from their perinatal counterparts in antigenic phenotype, morphology, cell cycle time, motility and time-course of differentiation *in vitro*.[7-10]

In this review we will discuss our attempts to understand the comparative biology of the precursor cells of developing and mature organisms. These studies have led us to propose functional distinctions between precursors which provide the basis for tissue formation during early development (e.g. neuroepithelial stem cells, embryonic stem cells), but which are not maintained in the animal throughout life, and those which are able to provide a source of new cells in tissues of mature animals. At least for the lineage we have examined, our studies have provided several novel insights into the origin and functional biology of stem cells of adult animals.

The O-2A^{perinatal} progenitor

The first step in our studies on glial development in the CNS was the discovery that cultures derived from white matter tracts of the CNS contained two distinct astrocyte populations, termed type-1 and type-2 astrocytes.[11] These two cell types could be readily distinguished from each other on the basis of morphology, antigenic phenotype and response to growth factors. Most importantly, we found that

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optic nerves of perinatal rats contained a population of glial precursors which did not express glial fibrillary acidic protein (GFAP) at the time of isolation, but which could be induced to become GFAP+ type-2 astrocytes by growth in tissue culture.

Subsequent studies on the precursors of type-2 astrocytes led to the discovery that these cells could also be induced to differentiate into oligodendrocytes.[5] Oligodendrocytic differentiation of O-2A progenitors occured when progenitors were grown in chemically-defined medium and did not require the presence of inducing factors. In contrast, astrocytic differentiation required the presence of appropriate inducing factors, at least one of which is found in fetal sera of a number of different species.

Initial studies on oligodendrocytic differentiation of O-2A progenitors isolated from optic nerves of perinatal rats presented the paradox that the cells we were studying were isolated at a time of maximal division of this lineage *in vivo*,[12,13] yet cells did not divide in tissue culture. Resolution of this paradox began with the discovery that cortical astrocytes promoted O-2A^{perinatal} progenitor division *in vitro*.[14] The astrocytes used in these studies express many of the properties of type-1 astrocytes of the optic nerve, which are the first identifiable glial cells to appear in the nerve.[15] The similarity of these two populations led us to suggest that type-1 astrocytes were responsible for supplying the mitogen(s) required to keep O-2A progenitors in division *in vivo*. Moreover, populations of O-2A^{perinatal} progenitors grown in the presence of purified cortical astrocytes were capable of undergoing extended division while also continuing to generate more oligodendrocytes,[14] a pattern of behaviour similar to that occurring *in vivo*. Thus, the failure of O-2A^{perinatal} progenitors to divide in our initial *in vitro* studies was due to the lack of necessary mitogens, which appeared to be supplied by another glial cell type of the optic nerve.

Further studies demonstrated that purified cortical astrocytes could also promote the correctly timed differentiation *in vitro* of O-2A^{perinatal} progenitors isolated from optic nerves of embryonic rats.[16] The molecular mechanism by which this timing is controlled remains a mystery, although all evidence to date indicates that it is the O-2A^{perinatal} progenitors themselves which are measuring elapsed time.[17,18] A potential linkage between the measurement of elapsed time by dividing cells and the control of differentiation has also been observed for fibroblasts and haematopoietic stem cells (for review see ref 19). In the case of O-2A^{perinatal} progenitors, it appears that this biological clock causes clonally related dividing progenitors to differentiate synchronously into oligodendrocytes within a limited number of cell divisions.[10,17,18] However, it is not yet known whether the mechanism which underlies this synchronous differentiation of clones of dividing cells is also responsible for the first appearance of oligodendrocytes in the rat optic nerve at the day of birth *in vivo*, or the equivalent time *in vitro*.

The effects of purified cortical astrocytes, and of type-1 astrocytes from the optic nerve, on O-2A^{perinatal} progenitor division *in vitro* appear to be mediated by platelet-derived growth factor (PDGF).[18,20-22] O-2A^{perinatal} progenitors exposed to either PDGF or astrocyte-conditioned medium exhibited a bipolar morphology, migrated extensively (with average migration rates of $24.6 \pm 5.6\,\mu m\,h^{-1}$) and divided with an average cell cycle length of 20 ± 6 h. PDGF was also as potent as type-1 astrocytes at promoting the correctly timed differentiation *in vitro* of embryonic O-2A progenitors into oligodendrocytes.[18] Moreover, antibodies to PDGF blocked the mitogenic effect of type-1 astrocytes on embryonic O-2A progenitor cells, causing these cells to cease division and to differentiate prematurely even when growing on monolayers of type-1 astrocytes. Thus, this single mitogen was able to elicit a complex behavioural phenotype from O-2A^{perinatal} progenitors which included normal functioning of the cellular mechanisms involved in the measurement of elapsed time. Interestingly, recent studies have indicated that neurons, which also promote division of O-2A^{perinatal} progenitors *in vitro*,[22,23] may also be a source of PDGF.[24,25] However, the specific contributions of either neuronal or astrocytic[26] production of PDGF to the development of the O-2A lineage *in vivo* is not yet known.

O-A^{adult} progenitors

To attempt to gain insights into the cellular mechanisms underlying regeneration of the oligodendrocyte population following demyelinating damage *in vivo*, we also initiated studies on O-2A progenitors of the adult CNS. In our initial studies, which again were focused on the rat optic nerve, we found that O-2A progenitors isolated from adult animals differed from their perinatal counterparts in several ways. When co-cultured with purified cortical astrocytes, O-2A^{adult} progenitors had a unipolar morphology *in vitro*,[7] whereas O-2A^{perinatal} progenitors were usually bipolar.[7,27] In

addition, O-2A^{adult} progenitors had a longer average cell cycle time *in vitro* than O-2A^{perinatal} progenitors (65 ± 18 h versus 18 ± 4 h),[7,20] migrated more slowly ($4.3 \pm 0.7\ \mu m\ h^{-1}$ versus $21.4 \pm 1.6\ \mu m\ h^{-1}$),[7,27] and take longer to differentiate (3-5 days versus 2 days for 50% differentiation).[7] Furthermore, O-2A^{adult} progenitors stimulated to divide by purified cortical astrocytes were O4$^+$ while dividing O-2A^{perinatal} progenitors were O4$^-$ (ref 7; I. Sommer, M. Noble, unpublished observations).

The appearance of adult-specific precursors in any lineage raises questions about their developmental origin. Are these cells derived from a common ancestor cell which, for example, initially gives rise to O-2A^{perinatal} progenitors, and then gives rise to O-2A^{adult} progenitors during later stages of development? Alternatively, are perinatal and adult precursor populations derived from two distinct ancestors, despite being specialized to produce similar terminally differentiated end-stage cells?

The continued presence of precursor populations in adult animals also raises questions about how such populations are maintained within any particular tissue throughout life. The maintenance of a precursor population throughout life is generally thought to be associated with the presence of a stem cell population which supplies new cells to the precursor pool for use in cell replacement following normal turnover or injury. For example, it has been suggested that the presence of proliferating O-2A progenitors in the adult animal requires the existence of a pre-progenitor, or stem cell, compartment in the O-2A lineage.[6] The requirement for a stem cell compartment to support the prolonged maintenance of dividing O-2A progenitors in the nerve is further indicated by the self-extinguishing nature of the O-2A^{perinatal} progenitor population. As described earlier, O-2A^{perinatal} progenitors grown *in vitro* in the presence of purified cortical astrocytes (as a source of PDGF)[14,20] generally divide and differentiate symmetrically, such that all members of a clonal family of cells synchronously differentiate into oligodendrocytes within a limited number of divisions.[17,18] It is clear that this mode of division and differentiation is incompatible with continued self-renewal of precursors throughout life, and it was thus not surprising to find that O-2A^{perinatal} progenitors are present only in small numbers in cultures prepared from optic nerves of 1-month-old rats[8] and are not detectable in cultures prepared from optic nerves of adult rats.[7]

Generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors

Analysis of the development of O-2A^{adult} progenitor cells in cultures derived from 3-week-old rats, the age when the relative proportion of perinatal to adult O-2A progenitors appears to be changing most rapidly *in vivo*,[8] has indicated that some O-2A^{perinatal} progenitor-like cells have the ability to generate O-2A^{adult} progenitor-like cells when co-cultured with purified cortical astrocytes.[10] These experiments were carried out by filming the behaviour of families of cells derived from single O-2A progenitors. Due to the simplicity of the optic nerve cultures, and our extensive characterization of the cell types found in these cultures, the morphological information provided in these films could be used to identify with great precision O-2A progenitors, and to distinguish between cells with *perinatal*- or *adult*-like phenotypes.

In seven individual time-lapse microcinematographic films, with a total analysis of 15 separate families of O-2A lineage cells, we found four examples of families in which (1) the founder cell gave rise at the first division to two cells with the characteristic morphology, cell-cycle length and motility of O-2A^{perinatal} progenitors and (2) subsequent to the first division, members of the family expressed the unipolar morphology, lengthened division times and slow migration rates typical of O-2A^{adult} progenitors.

Figure 1 depicts diagramatically the history of one of the families wherein O-2A^{perinatal} progenitor-like cells were seen to give rise to O-2A^{adult} progenitor-like cells. In this family the founder cell first generated two further O-2A^{perinatal} progenitor-like cells (cells a and b). The family branch represented by one of these progenitors (cell a) terminated, over two divisions, with the production of three oligodendrocytes (cells c, d and e, which were characterized by their multipolar morphology, lack of division and lack of migratory behaviour).[20,31] The other branch (cell b) first produced three further perinatal progenitor-like cells before all of these cells started to express longer cell cycle times and migration rates. By the next division, all of the motile and dividing members of this family expressed a unipolar morphology, a cell cycle length of > 40 h ($x = 45$ h) and a migration rate of $\leqslant 6\ \mu m\ h^{-1}$ ($x = 4\ \mu m\ h^{-1}$); see cells f, g and h in Figure 1. Similar observations were made in the other three families in which a *perinatal*-to-*adult*-transition was observed.[10]

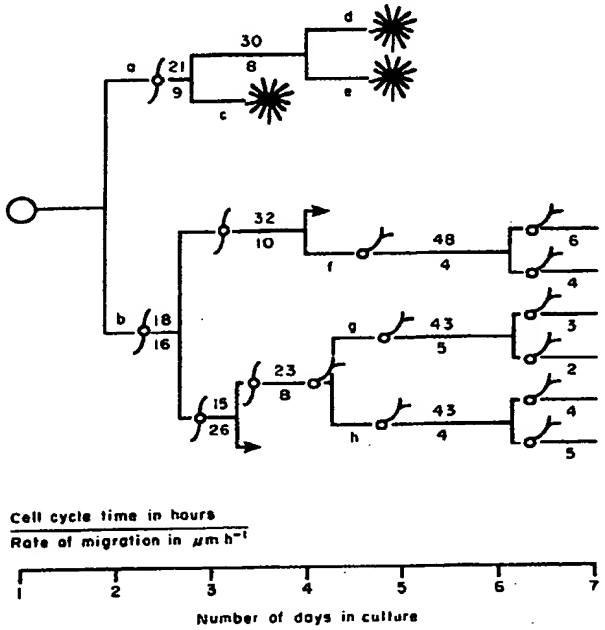

Figure 1. Bipolar O-2A^{perinatal} progenitor-like cells that divide and migrate at a fast rate give rise to unipolar O-2A^{adult} progenitor-like cells which migrate and divide more slowly. Fifteen clonal colonies of O-2A progenitors, stimulated to divide by cortical astrocyte-derived mitogens, were followed by time-lapse microcinematography. Within 15 colonies suitable for detailed analysis, four clear examples were found of O-2A^{perinatal} progenitor-like cells that were bipolar, were highly motile and had a short cell cycle time, which in the first division generated more O-2A^{perinatal} progenitor-like cells, and which eventually gave rise to cells which expressed the unipolar morphology, lengthened cell cycle time and slow migration rate of O-2A^{adult} progenitors. One of the families in which dividing O-2A^{perinatal} progenitor gave rise to O-2A^{adult} progenitor-like cells is represented diagramatically in the figure. The morphology of a progeny cell is indicated in the figure only when the cell was clearly bipolar, unipolar, or oligodendrocyte-like. Since some progeny cells moved out of the field of photography (depicted with an arrow) their fate could not be determined. The numbers above the lines are the cell cycle times in hours, while the numbers below the lines are the migration rates in $\mu m\ h^{-1}$. The transitions shown could not be ascribed to changes in the composition of the tissue culture medium, since all still cultures contained actively dividing and migrating O-2A^{perinatal} progenitor-like cells at the end of the filming period.

Our time-lapse observations suggest that the transition from *perinatal* to *adult* phenotype is not an abrupt one, in that generation of O-2A^{adult} progenitor-like cells may require two or more cell divisions, with the cells present after one division expressing cell-cycle times and motility characteristics intermediate between the *perinatal* and *adult* phenotypes. These results also are consistent with our previous studies on the characteristics of cells derived from optic nerves of 1-week to 1-month-old rats, in which we observed cells with phenotypes which could not be classified unambiguously as *adult*-like or *perinatal*-like.[8]

Extended self-renewal in the O-2A lineage is associated with the *in vitro* generation of O-2A^{adult} progenitors

To test further the hypothesis that O-2A^{adult} progenitors might be derived from O-2A^{perinatal} progenitors, we then serially passaged perinatal optic nerve cells over the course of 3 months. In these experiments, optic nerve populations containing O-2A^{perinatal} progenitors, but not O-2A^{adult} progenitors, were passaged onto fresh monolayers of purified and irradiated cortical astrocytes for up to six passages.

Serial passaging of O-2A progenitors derived from optic nerves of perinatal rats was associated with a shift in the progenitor population from entirely *perinatal*-like to predominantly *adult*-like, as judged by antigenic and morphological criteria and by changes in the population doubling times.[10] In the early passage cultures, dividing O-2A progenitor-like cells (identified by [^{3}H]- thymidine labelling, immunolabelling and autoradiography) expressed the bipolar morphology and A2B5$^+$O4$^-$ antigenic phenotype characteristic of O-2A^{perinatal} progenitor cells. In contrast, >80% of the dividing O-2A progenitors in the later passage cultures expressed the O4$^+$ antigenic phenotype characteristic of O-2A^{adult} progenitors, and 92% of these cells also expressed the characteristic unipolar morphology of O-2A^{adult} progenitors.[7] The rate of increase in the numbers of new progenitors and oligodendrocytes in these cultures also decreased significantly with increasing passage number, and fell from the 24 h doubling times characteristic of perinatal populations to approach the long doubling times characteristic of adult populations. In agreement with the increase in the average doubling time with increasing passage number, the proportion of progenitor-like cells which incorporated [^{3}H]-thymidine during a 24 h pulse decreased with successive passages.

The results of our serial passaging experiments were thus consistent with the hypothesis that O-2A^{adult} progenitors are derived from O-2A^{perinatal}

progenitors and further suggested that expression of the capacity for prolonged self-renewal in this lineage is associated with the appearance of O-2A^{adult} progenitors. The mechanism which might underly such self-renewal was suggested by observations that serial passaging was associated with a slight increase in the proportion of colonies which contained both oligodendrocytes and [^{3}H]-thymidine labelled O-2A progenitors, an observation examined in closer detail using cells derived from adult animals (see next section).

Characteristics of division and differentiation in colonies of O-2A^{adult} progenitor cells

As O-2A^{perinatal} progenitors are not detected at all in cultures derived from adult optic nerves, it is unlikely that generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors is the mechanism which allows maintenance of the *adult* progenitor in the nerve throughout life. The slight increase in the proportion of O-2A lineage colonies containing both oligodendrocytes and [^{3}H]-thymidine labelled O-2A progenitors, seen in our passaging experiments, raised the possibility that O-2A^{adult} progenitors might be able to divide and differentiate asymmetrically. Such a pattern of division and differentiation would allow these cells to give rise to more progenitors and generate oligodendrocytes at a slow rate. To examine this possibility under conditions which would allow cells to undergo several divisions, we analysed the composition and size of oligodendrocyte-containing colonies generated from O-2A^{perinatal} and O-2A^{adult} progenitors grown at clonal densities (<1 cell/30 mm^2) on monolayers of purified cortical astrocytes (to promote progenitor division).[7,14] As the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors (as would occur in cultures derived from 3-week-old rats) would have complicated analysis of these experiments, the optic nerve cells used in these experiments were obtained exclusively from newborn and adult rats. Colonies were examined after a length of time which would allow cells to undergo $\leqslant 6$ divisions and $\leqslant 10$ divisions, this being 6 and 10 days for O-2A^{perinatal} progenitors and 15 and 25 days for O-2A^{adult} progenitors, respectively.

Oligodendrocyte-containing colonies

As in previous experiments,[17] the composition and size of oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors were consistent with the view that the generation of oligodendrocytes by these cells is associated with symmetric division and clonal differentiation. Sixty-six percent of the oligodendrocyte-containing colonies examined on Day 10 consisted entirely of oligodendrocytes and, even as early as Day 6, the A2B5$^+$GalC$^-$ progenitor-like cells in mixed colonies were most frequently multipolar non-dividing cells (i.e. not labelled with [^{3}H]-thymidine) which appeared to have been visualized just prior to oligodendrocytic differentiation. Only 7% of the oligodendrocyte-containing colonies derived from *perinatal* progenitors and visualized on Day 6, and 14% of those visualized on Day 10, contained both oligodendrocytes and dividing progenitor cells (as judged by the incorporation of [^{3}H]-thymidine). Moreover, oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors clustered around sizes of 2, 4, 8, 16, 32, 64 and 128 cells/colony at 6, 8 and 10 days after plating, as expected when clonally-related cells divide symmetrically and differentiate synchronously.

Unlike the results obtained with O-2A^{perinatal} progenitors, the composition of oligodendrocyte-containing colonies derived from individual dividing O-2A^{adult} progenitors was consistent with the hypothesis that the generation of oligodendrocytes by these cells occurred by means of asymmetric division and differentiation.[10] Over 75% of the oligodendrocyte-containing colonies derived from individual O-2A^{adult} progenitors grown at clonal densities contained both oligodendrocytes (which generally do not divide in these tissue culture conditions)[7,14] and [^{3}H]-thymidine-labelled progenitors after both 15 and 25 days of *in vitro* growth, periods of time which would allow $\leqslant 6$ or $\leqslant 10$ average cell cycles for O-2A^{adult} progenitors.[7] The proportion of colonies which contained both oligodendrocytes and radiolabelled O-2A progenitor cells on Days 15 and 25 of *in vitro* growth was very similar, even though the average size of the oligodendrocyte-containing colonies continued to increase with time in culture (from a median value of 7 cells/colony on Day 15 to a median value of 11 cells/colony on Day 25). Only 10% of the colonies visualized on Day 25 consisted entirely of oligodendrocytes, and the remaining 14% contained oligodendrocytes and progenitors which were unlabelled by [^{3}H]-thymidine. In addition sizes of oligodendrocyte-containing colonies did not cluster at factors of 2 on either Day 15 or Day 25 of *in vitro* growth.

Oligodendrocyte-free colonies

Sixty-two percent (110/176) of the colonies derived from O-2A^{adult} progenitors contained no oligodendrocytes even after 25 days of *in vitro* growth. Oligodendrocyte-free colonies seen at this stage were generally small, and over 80% of these colonies (89/110) contained $\leqslant 16$ cells at Day 25. Consistent with the small size of many of these colonies, $<20\%$ (23/110) of the oligodendrocyte-free colonies contained any cells wich were labelled by a 20 h pulse with [^{3}H]-thymidine. In contrast, in colonies derived from O-2A^{perinatal} progenitors, only 30% (41/136) of the colonies were free of oligodendrocytes on Day 10 *in vitro*.

The O-2A^{adult} progenitor as a stem cell

While the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors provides a possible explanation for the origin of the *adult* cell, the lack of O-2A^{perinatal} progenitors in adult optic nerve[7] suggests that other mechanisms are involved in maintenance of a dividing population of O-2A^{adult} progenitor in the adult animal. Although it has been previously suggested[6] that the presence of such cells in the adult requires the existence of an ancestral stem cell, capable of generating O-2A lineage cells throughout life, several observations now raise the possibility that the O-2A^{adult} progenitors may themselves function as stem cells.

The first stem-cell like property of O-2A^{adult} progenitors derives from the observation that this population is maintained in the rat optic nerve as a dividing population seemingly throughout life (ref 6; G. Wolswijk, E. Abney, unpublished observations). O-2A^{adult} progenitor-like cells can be isolated from optic nerves during the first week after birth and such cells remain in the nerve for at least the first year of life, in contrast with O-2A^{perinatal} progenitors, which have largely disappeared from the optic nerve by one month after birth.[7,8] *In vitro* observations suggest that O-2A^{perinatal} progenitors would disappear from the nerve as a consequence of symmetric differentiation of most clones of cells into oligodendrocytes and differentiation of the remaining cells into O-2A^{adult} progenitors (and possibly type-2 astrocytes, although the *in vivo* existence of these cells is controversial; see refs 28-32).

The second stem-cell like property of O-2A^{adult} progenitors is their long (60-65 h) cell cycle times.[7-9] Our most recent studies [10] further suggest that the population of O-2A^{adult} progenitors may even contain a sizeable proportion of cells with cell cycle times in excess of 100 h. Examination of colonies developing *in vitro* over 25 days showed that the great majority (89/110) of these colonies contained $\leqslant 16$ cells after 25 days *in vitro*, and that only a small proportion (23/110) of these colonies contained cells which were labelled with a 20 h pulse of [^{3}H]-thymidine. Both of these results are consistent with the existence of O-2A^{adult} progenitors with very long cell-cycle times.

Also of potential relevance to the question of whether O-2A^{adult} progenitors express stem-cell like characteristics are our observations consistent with the view that these cells can undergo asymmetric division and differentiation *in vitro*. Unlike colonies derived from O-2A^{perinatal} progenitors, oligodendrocyte-containing colonies derived from O-2A^{adult} progenitors generally contained O-2A^{adult} progenitors which were labelled by [^{3}H]-thymidine, indicating that onset of differentiation in the *adult* progenitor-derived colonies was not associated with cessation of cell division in the whole colony. The capacity to undergo asymmetric division and differentiation is an important attribute of *bona fide* stem cells of adult animals.

A further stem-cell like feature displayed by *adult* progenitors grown *in vitro* was that a far higher proportion of oligodendrocyte-containing colonies than oligodendrocyte-free colonies contained O-2A^{adult} progenitors labelled with a 20 h pulse of [^{3}H]-thymidine (75 versus 20%; ref 10). Similarly, the onset of differentiation of epidermal stem cells into keratinocytes in any clone of cells is associated with an increased likelihood of finding cells engaged in DNA synthesis, in association with passage of stem cell progeny through a transit amplifying population of cells engaged in differentiation.[33]

Growth factor co-operation and self-renewal in the O-2A lineage

All of the research described thus far was carried out in cultures in which O-2A progenitor division was promoted either by purified cortical astrocytes or by PDGF (the progenitor mitogen secreted by these cells). However, we have also found that there are other developmental programmes which can be expressed by dividing O-2A progenitors. As will be discussed below, some of these findings may be of particular relevance to understanding the control of

precursor self-renewal and also to the elicitation from adult stem cells of a pattern of growth likely to be of importance in responding to tissue injury.

O-2A^{perinatal} progenitors division can be induced by exposure to cells to basic fibroblast growth factor (bFGF), but cells induced to divide by this mitogen were multipolar and showed little migratory behaviour.[34] In addition, cells induced to divide by bFGF had a cell-cycle length of 45 ± 12 h, in contrast with the 18 ± 4 h cell cycle length elicited by exposure to PDGF. These results indicate that PDGF and bFGF function in the O-2A lineage as modulators of differentiation as well as functioning as promoters of cell division. PDGF and bFGF also differ in their effects on oligodendrocytes themselves, in that only bFGF is able to promote division of these cells.[34-36]

The effect of bFGF on oligodendrocytic differentiation of O-2A^{perinatal} progenitors is currently a subject of controversy. In our initial studies, we found that O-2A progenitors exposed to bFGF differentiated prematurely to form oligodendrocytes.[34] In contrast, other investigators found that this same mitogen inhibited differentiation of purified O-2A^{perinatal} progenitors.[37] The several methodological differences between the two sets of studies (ranging from the source of progenitors to the methods of tissue culture) make it difficult to determine the reasons for these differing observations. Our more recent studies do however suggest that at least part of the discrepancy between the two sets of results may have been dūe to effects of other factors present in the cultures, and that bFGF does indeed inhibit oligodendrocytic differentiation of purified O-2A^{perinatal} progenitors.[46]

In respect to O-2A ^{perinatal} progenitors, the most intriguing results of our studies with PDGF and bFGF was the discovery that progenitors exposed simultaneously to these two mitogens continued to divide without differentiating into oligodendrocytes.[34] For example, cultures prepared from optic nerves of 19-day-old rat embryos began to generate oligodendrocytes after 2 days when established in the presence of PDGF alone,[18,34] yet remained oligodendrocyte-free even after 10 days of growth in the presence of PDGF + bFGF.[34] Further experimentation has demonstrated that O-2A^{perinatal} progenitors can be grown continually for a year or more *in vitro* as long as cells are continually exposed to both of these mitogens (S.C. Barnett, M. Noble, unpublished observations). O-2A^{perinatal} progenitors grown in this manner retain the ability to undergo oligodendrocytic differentiation when removed from the presence of both mitogens. We do not yet know whether O-2A^{perinatal} progenitors grown for extended periods in this manner will generate O-2A^{adult} progenitors.

The discovery that cooperation between growth factors can cause prolonged self-renewal of precursors revealed a previously unkown means of regulating self-renewal in a precursor population. Such cooperation may, however, represent a more general phenomenon, as indicated by the importance of growth factor cooperation in promoting the extended division *in vitro* of haematopoietic stem cells[38] and primordial germ cells.[47] It will be of considerable interest to determine the extent to which cooperation between different growth factors is responsible for eliciting particular aspects of stem cell behaviour.

Growth factor cooperativity and lesion repair

While it is difficult to determine the role (if any) played by PDGF/bFGF cooperativity during development, some of our most recent studies have suggested that such cooperativity may be of profound importance in the context of lesion repair. These studies have also revealed a further property of O-2A^{adult} progenitors of relevance in considering the stem cell-like behaviour of these cells.

We have recently found that simultaneous exposure of O-2A^{adult} progenitors to PDGF + bFGF converts many of these cells to a rapidly dividing and highly motile phenotype with a bipolar morphology and antigenic phenotype very similar to that expressed by O-2A^{perinatal} progenitors.[48] Thus, these cells can be induced to express a phenotype which seems likely to be of relevance to repair of demyelating lesions. These findings demonstrate that the molecular mechanisms which underlie the characteristic behaviour of O-2A^{perinatal} progenitors are not irreversibly inactivated with the generation of O-2A^{adult} progenitors, but are instead placed under the control of slightly different signalling processes than those which function in the perinatal cells. The finding that rapid cell division can be induced in O-2A^{adult} progenitors is consistent with observations that repair of virally-induced demyelination *in vivo* appears to be preceded by increases in the numbers of O-2A^{adult} progenitor-like cells.[39] In addition, studies in other laboratories have suggested an increased production of FGFs and PDGF following CNS damage.[40-42] It is particularly intriguing, however, that our studies also suggest that the ability of O-2A^{adult} progenitors to maintain a

rapidly dividing and migrating phenotype is not maintained beyond a small number of divisions, suggesting intrinsic limitations may exist in the extent to which these cells are capable of contributing to myelin repair.[48] Such a possibility is reminiscent of claims that MS lesions are initially repaired, but eventually become permanently demyelinated.

A revised view of the O-2A lineage

Figure 2 summarizes some of our current views about development of the O-2A lineage, in which the

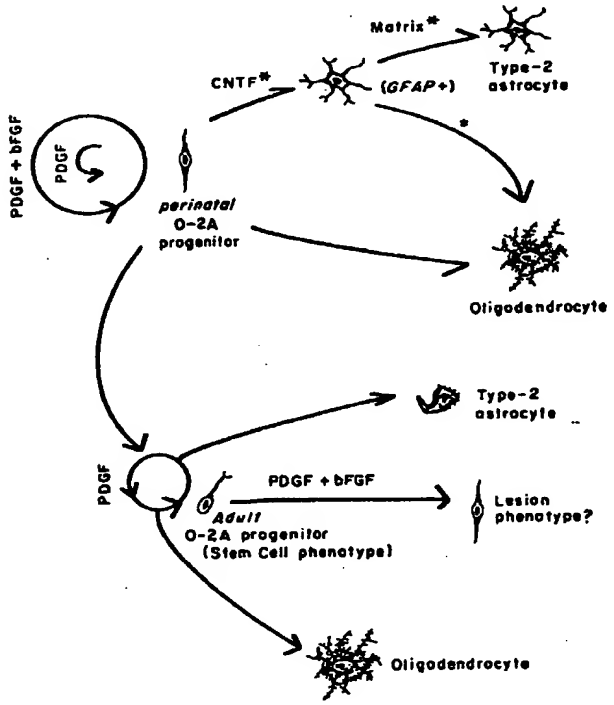

Figure 2. The O-2A lineage, as presently perceived in our laboratory. *Ciliary neurotrophic factor (CNTF) is thought to initiate astrocytic differentiation of O-2A progenitors, as detected by expression of glial fibrillary acidic protein (GFAP). Full differentiation of O-2A^{perinatal} progenitors into type-2 astrocytes, however, requires the additional presence of an unidentified extracellular matrix component (see refs 43, 44). Differentiation of O-2A^{adult} progenitors into type-2 astrocytes has not yet been extensively studied, although we have observed that the type-2 astrocytes generated following growth in serum-containing medium do not express the stellate morphology of type-2 astrocytes derived from O-2A^{perinatal} progenitors.[7] For more detailed discussion on other aspects of differentiation in this lineage see also refs 21, 29, 45.

population of O-2A^{perinatal} progenitors is now seen as tripotential and capable of giving rise to oligodendrocytes, type-2 astrocytes and O-2A^{adult} progenitors. Our studies suggest that O-2A^{perinatal} progenitors express the properties of true progenitor cells, in that these cells generally express a limited life-span before undergoing differentiation (at least when stimulated by purified cortical astrocytes or PDGF). However, a previously unanticipated differentiation pathway which appears to be open to O-2A^{perinatal} progenitors is to give rise to a new generation of precursors, these being the stem cell-like O-2A^{adult} progenitors. The apparent development of O-2A^{adult} progenitors, with stem cell-like characteristics, from a rapidly dividing perinatal population differs significantly from the pattern of development seen in other lineages, where slowly dividing stem cells (of developed, rather than developing, tissues) have been seen to give rise to rapidly dividing progenitors (for review see ref 33). However, no other studies have focused on the origin of potential stem populations in the manner in which we have.

A general hypothesis on the origin of stem cells, which we believe emerges from our studies, is as follows: precursors which function during early development express properties required for cells participating in the initial creation of a tissue. Such properties are inappropriate at later developmental stages, at least for some tissues. In such instances, the early precursors give rise to a second generation of precursors with properties more appropriate to later development, as well as to terminally differentiated end-stage cells. It is this second generation of precursors which represent the stem cells of adult animals. Unfortunately, it will first be necessary to be able to distinguish unambiguously between fetal (or perinatal) and adult precursor cells in other tissues before it can be determined whether phenomena similar to those observed in the O-2A lineage also occur in other lineages.

In regards to the O-2A lineage itself, there are many challenging questions which remain unanswered. It will first be important to determine whether the process we have described for the O-2A lineage of the optic nerve occurs in all regions of the CNS. On a more fundamental level, it will be necessary to determine the relationship between the symmetric and asymmetric O-2A^{perinatal} progenitors. Are these two cell types distinct from the beginning of their existence, is one cell type the ancestor of the other, or do they represent two possible differentiation pathways of a still earlier ancestor cell? Moreover,

although it seems likely that it is the asymmetrically behaving O-2A^{perinatal} progenitors which eventually give rise to 0-2A^{adult} progenitors, the mechanism which causes the earlier cells to generate cells of an *adult* phenotype is a mystery. At present, we know that O-2A^{perinatal} and O-2A^{adult} progenitors exposed to platelet-derived growth factor (PDGF) each express their characteristic morphologies, migratory properties and cell cycle lengths.[9,20] It will be an important challenge to define the molecular alterations which allow a single cell-signalling molecule to elicit such different behaviours from these two precursor populations, and to determine whether these alterations are alone sufficient to convert O-2A^{perinatal} progenitors into O-2A^{adult} progenitors. It is also interesting that this replacement of an O-2A^{perinatal} progenitor population by an O-2A^{adult} progenitor population *in vitro* is at least superficially similar to that which occurs *in vivo* (albeit over a slightly shorter time-scale than that seen *in vitro*). The ability to reproduce such a conversion in tissue culture will facilitate future studies on molecular mechanisms which might be involved in the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors. Finally, the O-2A^{adult} progenitor cell may offer a suitable model system for probing the molecular mechanisms involved in the generation of asymmetric division and differentiation.

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